ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTES OF HEALTH

1967 - 1968

NATIONAL INSTITUTE OF ARTHRITIS
AND METABOLIC DISEASES

U.S. NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

" Report of program activities

ANNUAL PROJECT REPORT

1967 - 1968

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THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL REPORT

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ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR

National Institute of Arthritis and Metabolic Diseases

Fiscal Year 1968

by

Dr. J. E. Rall

- - - -

In the intramural program of NIAMD we have said "Good-bye" regretfully to Drs. Leon Heppel and Bruce Ames who have moved to Cornell University and the University of California in Berkley respectively, and to Dr. Keresztesy who has retired. We have been fortunate in persuading Dr. Herbert Sober from the National Cancer Institute to take over the Laboratory of Nutrition and Endocrinology, where he will devote most of his energies to expanding the scope of the large scale laboratory. Dr. Ashwell has assumed responsibility for the Laboratory of Biochemistry and Metabolism, and Dr. Robert Martin for the Section on Microbial Genetics in the Laboratory of Molecular Biology. The Laboratory of Experimental Pathology has been strengthened by the addition of Drs. Ned Feder and Vincent Marchesi as Chiefs of the Sections on Biophysical Histology and Pathologic Anatomy respectively.

Work in this Institute continues to span the length of medical research from the most fundamental aspects of mathematics and chemical physics to studies on the therapeutic effectiveness of new drugs used to treat arthritis. The reports which follow summarize the work in each Laboratory and Branch.

Studies on Folic Acid

The current investigation on dihydrofolic reductase in conjunction with the previously reported effects of organic mercurials and iodine suggest that high concentrations of urea and thiourea stimulate the activity of chicken liver dihydrofolic reductase by inducing a specific activation of the enzyme. It is assumed that the primary effects of these high concentrations of urea and thiourea is to induce a change in the conformation of the protein yielding a catalytically more favorable enzyme. This activated state may either enhance the transfer of the hydride ion from TPNH to dihydrofolate or facilitate the binding of the two substrates.

The lack of these activating effects when either DPNH is utilized as the reducing agent or when folate is utilized as the substrate suggests that binding of TPNH or TPNH plus dihydrofolate are important factors in this activation phenomenon. Thus binding of one or both of the two substrates may be required to allow the appropriate concentration of urea or thiourea to induce the specific conformational change in the enzyme.

This hypothesis is consistent with the stability studies, which demonstrate that in the absence of both substrates the activity of chicken liver dihydrofolic reductase is rapidly destroyed by the activating concentrations of urea and thiourea. This marked lability is to be contrasted with the striking stabilization observed in the presence of either usual substrate, particularly TPNH. DPNH is completely inert as a stabilizing agent. That this lack of stabilization may be related to the absence of the additional phosphate present in TPNH is suggested by the observation that when DPNH is utilized as the substrate, inorganic phosphate becomes an effective inhibitor. On the other hand, in the presence of TPNH, inorganic phosphate possesses no inhibitory properties.

Although dihydrofolic reductase from yeast is quite similar to that in chicken liver with respect to coenzyme requirements and approximate molecular weight, the significant differences observed suggest that it is more related to the chicken liver enzyme in the activated state than the native chicken liver enzyme. Purification has been limited by marked lability. Thus far, stabilization occurs only in the presence of high concentrations of EDTA or 20% glycerol. Reagents which result in the marked activation of chicken liver enzyme inhibit or destroy the yeast enzyme. (Dr. B.T.Kaufman)

Since folic acid as such most probably does not occur in nature, investigations are in progress to isolate and determine the chemical and biological properties of the more complex naturally occurring forms. The folic acid derivative found in yeast has been partially purified by means of extraction with boiling ascorbic acid, adsorption and elution from charcoal, precipitation as a barium salt and chromatography on DEAE cellulose. At this point the material appears to be 30 to 50% pure. Two related compounds appear to be present. Microbiological properties suggest that the major compound may be N^5-methyl-tetrahydropteroyl-p-aminobenzyl-heptaglutamate. (Drs. B. T. Kaufman, J. C. Keresztesy and Mr. H. Bakerman)

In view of the rapid destruction of the complex folates in isolated tissues, the structure and functions of the enzymes resulting in this process are under investigation. The enzyme responsible for the degradation of the folate polyglutamates has been partially purified from an acetone powder of chicken pancreas. With the availability of the partially purified complex folate from yeast, a relatively specific enzymatic assay has been devised. Preliminary evidence suggests that a metal may be necessary for stability and enzyme activity. This may be magnesium. (Dr. B. T. Kaufman)

Large-scale Processing of Biological Materials

The large-scale laboratory unit has continued to improve its effectiveness by assisting N.I.H. investigators in carrying out procedures when large volumes or quantities of materials are involved. Currently, a 300 gallon fermentor is being installed due to the great demand for the growth of microorganisms utilizing the two 100 gallon fermentors. During the past year, more than 130 fermentations were run, yielding kilogram amounts of over 13 varities of bacteria (batch #1000 was completed on March 21, 1968). In addition to harvesting the cells, the supernatant culture fluid from the growth of several bacteria was processed for an extracellular enzyme.

Other large scale processing activities were the lysis of cells and the extraction and partial purification of large amounts of enzymes, nucleic acids, parathyroid hormone, folic acid derivatives, alkaloids, terpenes and glycosides from bacteria, animal tissues and plants. (Drs. D. L. Rogerson, J. C. Keresztesy and B. T. Kaufman)

Studies in Experimental Nutrition

The utilization of germfree animals continues to serve as an experimental approach in order to differentiate between the effects of a particular nutritional state and the effects due to the bacterial flora characteristic of the experimental animal. Thus it has been observed that iodine metabolism and the development of goiter is influenced by the nature of the bacteria of the intestinal tract. In addition, preliminary results indicate that the early death in vitamin A deficiency may be due to the inability of the animal to combat infection. Use of germfree animals is making it possible for the first time to study the direct effects of a severe vitamin A deficiency. (Dr. F. S. Doft and Mr. E. G. McDaniel)

Insulin

Radioimmunoassay (RIA) of Insulin. In connection with our studies on induction of diabetes, we have made a limited number of determinations of insulin concentrations in plasma and pancreas, using the double antibody method of RIA. This double antibody method takes several days and requires a second antiserum which is expensive and difficult to procure. Hence, for the past few months much time has been spent perfecting Dr. Kevin Catt's solid phase RIA which eliminates the second antibody reaction. We have found that plastic disposable microtiter plates which are used in immunology and contain 96 wells, are excellent for this purpose. The wells have a volume of 0.2 ml. These may be coated with the insulin antibodies by flooding the plate with anti-insulin serum. The plate is then washed several times to remove unbound anti-insulin serum. The test solutions and labelled insulin are then added and the plate is placed in a refrigerator overnight. In the morning the plate is rinsed with tap water several times to remove unbound insulin. The plate is then easily cut apart with scissors and the individual wells are placed in the usual tubes for counting of the bound radioactive insulin. The assay is completed in 24 hours. Satisfactory results can be obtained the same day with only 3 or 4 hours of incubation at room temperature but overnight incubation in a refrigerator gives more accurate results. The cost of materials and reagents for each assay is reduced to 20% and the labor by more than 50%. (Dr. R. W. Bates)

Experimental Diabetes

Induction of Diabetes with Exogenous Hormones. Growth hormone and ACTH act synergistically to produce diabetes in partially pancreatectomized rats. When ACTH was replaced by various corticoids, hydrocortisone was found not to be synergistic with GH, whereas dexamethasone and corticosterone showed partial synergism. (Dr. R. W. Bates)

Prophylaxis of Diabetes Induced by Endogenous Hormones.

(1) Prophylactic Effect of Insulin. Every partially (80%) pancreatectomized ($\overline{P}$) rat that bears the transplantable pituitary tumor MtT-F4, which makes GH and ACTH, will develop diabetes within one week. Such rats were treated daily for 21 days with an amount of insulin that prevented or permitted minimal glucosuria. This treatment prevented development of permanent diabetes in about three-fourths of the rats.

(2) Prophylactic Effect of Anti-adrenal Drugs. Recently the drugs Metopirone and Elipten have been shown to interfere rather completely with certain pathways in the synthesis of adrenal steroids and thus reduce the levels of adrenal steroids in the body. Since adrenalectomy will prevent development of diabetes in about half of the 80% $\overline{P}$ rats with MtT, studies were made of the potentiality of Metopirone and Elipten as prophylactic agents for the prevention of diabetes. When 0.5% Metopirone or 0.1% Elipten were added to the diet of 80% $\overline{P}$ rats with MtT, the onset of diabetes was delayed for one to two weeks, but by 21 days the hormonal stimulus was so great that about half of the rats became severely, if not permanently, diabetic. When both drugs were added to the diet in the same concentration, the protection was the same, about 50%. At the end of the 21 day treatment, the concentration of corticosterone in plasma and adrenals was reduced to minimal normal control levels by Elipten and by the mixture. The total amount of corticosterone in the adrenal, however, was still roughly normal because of the 3 to 6-fold enlargement of the adrenal. Elipten and Metopirone had a demonstrable prophylactic effect in reducing the onset and extent of development of diabetes, presumably by reducing adrenal steroid production by the ACTH produced by the tumor.

(3) Effect of Elipten and Metopirone on Splanchnomegaly. Addition of the anti-adrenal drugs to the diet permitted the enlargement of the liver, kidneys and heart which occurs in MtT tumor bearing rats. This is in contrast

to the effect of adrenalectomy which has previously been shown to prevent completely splanchnomegaly. This new finding suggests again that some unrecognized product of the adrenal cortex is required for splanchnomegaly. Perhaps these are 18-OH corticoids, on the basis of the paper by Francois, Bates and Johnson. (Dr. R. W. Bates)

Studies of Ketosis in Hypophysectomized-Pancreatectomized Rats. Earlier studies showed that dexamethasone caused very severe ketosis in fasting hypophysectomized-pancreatectomized ($\overline{\text{HP}}$) rats when the hormone was given one hour after hypophysectomy but not when given seven days later. It was then postulated that the ketogenic action of the glucocorticoid might require the presence of a hypophyseal factor which disappeared from the animal during the first week after hypophysectomy. Recently it was found that dexamethasone caused severe ketosis in $\overline{\text{HP}}$ rats deprived of the pituitary gland for one week if either growth hormone or ACTH gel was given with the glucocorticoid; neither pituitary hormone was ketogenic when given singly. Dexamethasone also caused ketosis if the animals were pretreated 48 and 24 hours before with growth hormone. These findings indicate that glucocorticoids are ketogenic in $\overline{\text{HP}}$ but only if the animals are conditioned by either growth hormone or ACTH. The nature of this phenomenon is under study. (Drs. C. M. Clark and R. O. Scow)

Ketosis of Fasting. The effect of insulin on free fatty acid (FFA) turnover (using ^{3}H-oleic acid) and ketosis in the fasting rat was studied *in vivo* using a technique which allows continuous blood sampling for two hours. Blood loss is replaced by continuous transfusion. Acute insulin deprivation, induced by D-mannoheptulose and guinea pig anti-insulin serum, increased plasma FFA concentration, FFA production and FFA uptake, and at the same time caused the development of ketosis. Injection of insulin, in both large and small doses, rapidly decreased FFA production, lowered plasma FFA concentration and simultaneously abolished ketosis of fasting. No effect on ketosis could be obtained without a concurrent effect on FFA production. Studies thus far suggest that the effect of insulin on ketosis is mediated through its effect on FFA production and plasma FFA level. Studies of the effect of insulin on ketone body production and utilization *in vivo* are currently underway. (Drs. F. Bieberdorf and R. Scow)

Hormonal and other factors that affect ketone body production are also being studied in the perfused isolated rat liver. It has been found that hepatic ketone body output is closely dependent on plasma FFA concentration and that insulin has no effect in the liver on ketone body production. These findings complement those made in the intact rat. (Drs. C. M. Clark and R. O. Scow)

Studies on ketosis of fasting in pregnant rats have shown that the level of insulin in plasma is not low enough to explain increased lipid mobilization and hyperketonemia found in these rats. The possibility that a relative resistance to insulin exists in pregnant rats was investigated. Insulin administration (1.0 or 0.2 units) lowered the plasma concentrations of glucose, free fatty acids (FFA), ketone bodies and triglycerides in fasted pregnant and control rats. The plasma FFA concentration in the pregnant rats, which is higher than that in non-pregnant rats, did not fall to the low values seen in the control rats, even at 3 hours after insulin. This finding suggested that insulin may not completely suppress lipolysis in adipose tissue of fasting pregnant rats. In vitro experiments confirmed that fatty acid release in adipose tissue from fasted pregnant rats was greater than that from fasted controls and that it was not decreased by insulin injection. The data suggest that the lipolytic mechanism in adipose tissue is resistant to insulin during pregnancy. Since growth hormone and glucocorticoids are both needed for development of ketosis in the pregnant rat, it is possible that they are responsible for the insulin resistance seen. (Dr. S. S. Chernick)

During the course of the above studies, a new micro-procedure for measuring acyl glycerol was developed. It involves hydrolysis of acyl esters with tetraethanol ammonium hydroxide, which leaves phosphate esters intact, and the liberated glycerol is assayed enzymatically in a fluorimeter. (Dr. S. S. Chernick)

Metabolism of Adipose Tissue

Effects of Lipolytic Hormones on Adenyl Cyclase of Fat Cell "Ghosts" It was previously reported that the lipolytic hormones ACTH, epinephrine, and glucagon stimulate adenyl cyclase activity of fat cell "ghosts." A study of the hormone--adenyl cyclase interaction was undertaken to explore whether one or more enzymes are involved and to obtain some information on the primary site of action of these hormones and perhaps on their primary mechanism of action. It was found that the stimulatory effects of the three hormones when tested together were not additive when maximally stimulating concentrations were used. It was concluded that only one enzyme is affected by them.

Propranolol, an inhibitor of the β adrenergic effect of epinephrine, inhibited only the stimulation obtained with the catecholamine. The activation achieved with the polypeptide hormones, ACTH and glucagon, was not affected by the β blocker. Combination of the hormones in sets of two at half concentrations caused a higher activation of adenyl cyclase than when the single hormones were added near their K_a. These experiments suggest that each hormone acts via a specific receptor site, that adenyl cyclase is not the primary site of action of these hormones, and that hormone specificity is determined by receptor sites present in the outer plasma membrane. The K_a found for ACTH, glucagon, and epinephrine are 3.0×10^{-7}M, 0.9×10^{-7}M, and 60×10^{-7}M, respectively. The optimal pH for enzyme activity, as well as for hormone stimulation (determined with ACTH) is 8.2. ATP concentration curves at constant Mg:ATP ratios are sigmoid in shape. Addition of ACTH accentuates the sigmoidal shape, confirming the idea that the hormones act allosterically on adenyl cyclase activity. (Dr. L. Birnbaumer)

Effect of Insulin on Chylomicron Uptake by Perfused Adipose Tissue. Previous studies in this laboratory showed that during the removal of chylomicron glycerides from blood by perfused adipose tissue, about 3/4 of that removed was hydrolized to glycerol and free fatty acids and 1/4 was transferred intact to the tissue. The studies also showed that 70% of the fatty acids released from chylomicron glycerides, by the action of lipoprotein lipase, were incorporated into tissue glycerides. The effect of fasting and insulin lack on chylomicron uptake has now been studied. It was found that the amount of fatty acids split off from chylomicrons and incorporated into tissue lipids was 50% less in perfused fat pads of pancreatectomized and fasted rats than in those of fed rats. It was also observed that infusion of insulin, at a concentration 100 μU/ml blood, restored immediately this process to normal in perfused adipose tissue of pancreatectomized rats. Since preliminary results suggest that hydrolysis of chylomicron glycerides is rate limiting, these findings indicate that insulin may have a direct and immediate effect on lipoprotein lipase activity in adipose tissue. The site and nature of this action is now under study. (Drs. M. Hamosh and R. O. Scow)

Fatty Acid Metabolism in Perfused Adipose Tissue. Free fatty acids (FFA) are removed from blood, esterified and stored by adipose tissue as triglyceride. Biochemical studies have shown that the esterification process occurs in the particulate fraction and that there might be several pools of fatty acids in the tissue. It has been suggested that newly synthesized glyceride is transferred from site of synthesis to site of storage in the form of membrane-enclosed lipid droplets. The mechanism of FFA transfer from blood to fat cell is not known.

These processes were studied with electron microscope radioautography in perfused adipose tissue taken from starved-refed rats. The fat pads were perfused with diluted blood containing insulin (2 mU/ml) and glucose. The tissues were infused intraarterially with ^{3}H-oleic acid (complexed with albumin) for various times, ranging from 0.3 to 5.3 minutes, and then fixed immediately with cold glutaraldehyde which stopped at once all metabolic processes. Later, part of the tissue was used for chemical analyses and part for electron microscopy.

Since the vascular bed was not flushed of radioactive oleic acid, about 1/3 of the radioactivity in the tissue was present as FFA, or unesterified fatty acids, and the rest was present as esterified fatty acids. The labeled free fatty acids in the tissue were found associated with albumin. Whether the albumin-FFA complex was intravascular or extravascular was not determined, but it seems unlikely that it would be present within the fat cells.

More than 3/4 of the newly esterified fatty acid, even at 18 seconds, was in the triglyceride fraction and about 1/4 in diglyceride; the phospholipid fraction contained less than 1%. The rate of esterification of oleic acid was between 0.1 and 0.2 μEq/ (min x g).

The radioautographic reaction, as determined with the electron microscope, was seen at all times predominantly over fat cells and rarely over connective tissue or endothelial cells. The silver grains in fat cells were seen mostly over lipid droplets of all sizes, much less frequently associated with endoplasmic reticulum and membrane-bound vesicles, and seldom with mitochondria. The relatively large size of silver granules, however, made localization of radiation source to specific intracellular organelles very difficult. No evidence was seen that triglyceride is transported in small lipid droplets.

The findings indicate that free fatty acids are rapidly transferred from the blood stream to the site of glyceride synthesis within the fat cell and the newly synthesized glycerides are then transferred directly to the nearest lipid droplet. The manner in which FFA and newly synthesized glycerides are transported within adipose tissue remains unknown. (Dr. R. O Scow in collaboration with Drs. Olga and Yechezkiel Stein of Hebrew University-Hadassah Medical School, Israel).

Studies of Vitamins A and E

In studies of vitamin E activity as related to chemical structure, the vitamin E biopotency of dl,N-methyl-β-tocopheramine in the rat fertility assay was found to be 90% that of dl,α-tocopherol. When N,N-dimethyl-γ-tocopheramine, reported to be biologically active in the chick, was fed to rats, it was partially demethylated as indicated by the presence of both dimethyl and monomethyl-γ-tocopheramine in liver in a ratio of 7:1. Since the monomethyl-β- and γ-tocopheramines were shown previously to be demethylated to their corresponding tocopheramines, these studies show that the α-tocopherol structure is not specific for optimal vitamin E activity. (Dr. J. G. Bieri)

Studies of the effect of vitamin A on enzymes have revealed that L-gulono-γ-lactone oxidase activity in liver is greatly reduced in vitamin A deficient rats. The possible complicating effects on this enzyme of the reduced food intake of deficient rats are being evaluated. In other studies of the role of vitamin A in infection, germ-free rats maintained on a vitamin A-free diet grew normally for about six weeks, then leveled off in weight and developed a mild incoordination. Conventional vitamin A deficient rats all died in 6-7 weeks but the germ-free animals have survived for five months. Several deaths in these rats did not appear to be due to causes related to the absence of vitamin A, e.g., intestinal strangulation and bladder stones. Other findings at autopsy, such as testicular degeneration and corneal and retinal lesions indicate that vitamin A is required for maintenance of some specialized tissues but is not essential for suvival in the absence of bacterial injection. (Dr. William Rogers)

Regulation of Liver Cytochromes

Cytochrome b isolated by existing procedures has no enzymatic activity. Two isolation techniques were developed which gave products with considerable oxidizing ability. In one, treatment of crude cytochrome b with a bacterial protease gave a soluble product with 10-20% of theoretical activity. A second procedure utilizing Sephadex chromatography yielded cytochrome b with 40-80% activity and which was estimated to be 50% pure. This is the most highly purified cytochrome b ever prepared. (Dr. Jesse Williams, Jr.)

Lipoprotein Biosynthesis

Development of a procedure for collecting intestinal lymph has made studies of the origin and transport of endogenous lymph lipids possible. This has revealed that the intestine produces β-lipoprotein and triglycerides. The intestinal synthesis of β-lipoprotein is unaffected by dietary orotic acid, in contrast to the almost total inhibition of β-lipoprotein production in liver by orotic acid. About 10% of plasma triglyceride in the rat on a fat-free diet is from the intestinal mucosa, and the fatty acids of this triglyceride are obtained from mucosal phospholipids. In the rat fed a fat-free diet, intestinal lymph contained chylomicrons similar in size and properties to those found in lymph after a fatty meal. This study provides the first clear picture of fat metabolism by the intestine in the absence of dietary fat. (Dr. H. G. Windmueller)

Serial No. NIAMD-LNE-1
1. Nutrition and Endocrinology
2. Vitamin Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Folic Acid

Previous Serial No.: Same

Principal Investigators: Drs. B. T. Kaufman and J. C. Keresztesy

Other Investigators: Mr. H. A. Bakerman, Miss R. C. Gardiner and Mrs. M. K. Romine

Cooperating Units: None

Man Years:
Total: 6-1/2
Professional: 4
Other: 2-1/2

Project Description:

Objectives: To isolate and determine the biochemical properties of enzymes concerned with the metabolism of folic acid especially dihydrofolic reductase from chicken liver and yeast and the folate conjugase enzyme from chicken pancreas and to isolate, determine the chemical structure and biological activity of the naturally occurring forms (polyglutamates) of folic acid in microbial and animal cells.

Methods Employed: Standard chemical and biochemical techniques appropriate for purification and fractionation of vitamins and proteins are utilized in these projects in conjunction with various methods of enzymatic and microbiological analyses.

Major Findings: Chicken, Liver, Dihydrofolic, Reductase: The discovery that the marked susceptibility of this enzyme to destruction by certain proteolytic enzymes is completely eliminated by the presence of TPNH has served as the basis for an improved purification procedure. Thus sufficient amounts of homogenous enzyme will soon be available for physiochemical characterization.

Detailed kinetic analyses of the dramatic stimulation of enzyme activity by 4 M urea or 1.6 M thiourea suggests that TPMH is an integral component of the activation process in this case as well as the activation of the enzyme by organic mercurials and inorganic iodine. A scheme is proposed whereby urea or thiourea disrupt the tertiary structure of the native enzyme and allow it to assume a more unfolded conformation which is characterized by a marked increase in lability. In the presence of TPNH, this second form of the enzyme partially refolds to assume the more stable activated conformation. Thus DPNH which is a good coenzyme for the reductase cannot induce this secondary refolding and these activations are not observed when DPNH is utilized as a coenzyme. (Dr. B. T. Kaufman and Miss R. Gardiner)

Yeast Dihydrofolic Reductase: Although the enzyme from yeast is quite similar to that in chicken liver with respect to coenzyme requirements and approximate molecular weight the significant differences observed suggest that it is more related to the chicken liver enzyme in the activated state than the native chicken liver enzyme. Purification has been limited by marked lability. Thus far, stabilization occurs only in the presence of high concentrations of EDTA or 20% glycerol. Reagents which result in the marked activation of the chicken liver enzyme inhibit or destroy the yeast enzyme. (Dr. B. T. Kaufman and Miss R. Gardiner).

Naturally Occurring Forms of Folic Acid: The unknown complex folic acid derivative found in yeast has been partially purified by means of extraction with boiling ascorbic acid, adsorption and elution from charcoal, precipitation as a barium salt and chromatography on DEAE cellulose. At this point the material may be 30 to 50% pure. Two related compounds appear to be present. Microbiological properties suggest that the major compound may be N^5-methyl-tetrahydropteroyl-p-aminobenzyl-heptaglutamate. (Drs. B. T. Kaufman, J. C. Keresztesy and Mr. H. Bakerman).

Chicken Pancreas Conjugase: The enzyme which degrades the compex folates has been partially purified from an acetone powder of chicken pancreas. With the availability of the partially purified complex folate from yeast, a relatively specific enzymatic assay has been devised. However, further progress in purification has been limited by the lack of stability of the partially purified enzyme preparation. Preliminary evidence suggests that a metal may be necessary for stability and enzyme activity. This may be magnesium. (Dr. B. T. Kaufman and Mrs. M. Romine).

Significance to Bio-Medical Research and the Program of the Institute: Dihydrofolic reductase is not only a key enzyme in the metabolism of folic acid it is also of major importance in the synthesis of thymidine and the chief site of action of the anti-folic drugs. Thus the understanding of the structure and function of this enzyme would be of basic importance in both normal metabolic interrelationships and cancer chemotherapy. Folic acid as such most probably does not occur in nature. Thus the knowledge of chemical structure and biological properties of the more complex natural form will result in a better understanding of the biochemistry of this vitamin. In view of the rapid degradation of the natural folates in isolated tissues, the structure function of the enzymes responsible for this process (conjugases) may be of importance in understanding of the metabolism of these complex forms of folic acid.

Proposed Course of the Project: Investigations will continue on mechanism of activation of chicken liver dihydrofolic reductase from chicken liver by organic mercurials and iodine. The lack of such effects on the similar enzyme from yeast will be examined in relation to the known properties of the chicken liver enzyme. This will require further purification of the yeast enzyme. Studies on purification and biochemical properties of the folic acid conjugase from chicken pancreas will be continued. The purification of the yeast folate will continue until the pure substance is obtained in order that its exact chemical structure is elucidated.

Publications: None

Serial No. NIAMD-LNE-2
1. Nutrition and Endocrinology
2. Vitamin Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Large-scale processing of biological materials

Previous Serial No.: Same

Principal Investigators: Mr. D. L. Rogerson, Jr., Dr. J. C. Keresztesy and Dr. B. T. Kaufman

Other Investigators: Mrs. M. K. Romine and Mr. H. A. Bakerman

Cooperating Units: None

Man Years:
Total: 5-1/2
Professional: 3
Other: 2-1/2

Project Description:

Objectives: Large amounts of natural materials, such as bacteria, animal tissues, cells, blood, urine and plant materials require facilities for their production and processing in order that they may be studied under standard laboratory conditions. Facilities for such large-scale processing are provided under this project.

Methods Employed: When called upon for large-scale processing by any NIH investigator, methods are designed for the scaling-up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc. so that the processing can be carried out effectively and safely. Two 100 gallon fermentors are utilized for growing microorganisms under a variety of conditions. The bacterial cells are harvested by means of a regular or large scale Sharples centrifuge. Cells are either supplied as a paste or further processed to yield cell free suspension by rupture with a Gaulin Homogenizer. Currently, a 300 gallon fermentor is being installed along with a 360 gallon/hour deionized water source. In addition a second large scale Sharples AS-16P centrifuge is being installed to handle the increased production of microorganisms.

Standard methods of microbiological analysis are utilized to evaluate the sterility of the fermentation conditions, the nature and condition of the innoculum supplied by the investigator and bacterial purity of harvested cells.

Major Findings: During the past year 133 kilogram quantities of 13 varieties of bacteria were produced (Batch #1000 was completed on March 21, 1968). The microorganisms produced during this period consisted of regular and mutant strains of Aerobacter aerogenes, Bacillus megatherium, Bacillus subtilis, Clostridium pasteurianum, Escherichia coli, Proteus mirabilis, Pseudomonas acidivorans, Salmonella typhimurium, Serratis marcescens, and streptococcus lactic. In addition to harvesting the cells, the supernatant culture fluid from the growth of Bacillus subtilis was processed for an extracellular enzyme. Both the cells and supernatant culture fluid from Streptococcus lactis were extracted for the antibiotic Nisin.

Large scale processing activities consisted of extraction of phosphodiesterase from 8 kg of calf spleen, fatty acid activating enzyme from 6 kg bovine liver, RNA from 30 lbs. of Baker's yeast, parathyroid hormone from 5 kg parathyroid glands, and the salts of unknown folic acid derivations from large amounts of Baker's yeast. Alkaloids, terpenes, and glycosides were extracted from a variety of plant tissues such as leaves, seeds and stems of solanum xanthocarpum and tecoma stans. The large scale equipment was utilized to prepare lyophilized iodine-free diets and approximately 60 gallon of hydroxyapatite.

Significance to Bio-medical Research and the Program of the Institute: With this laboratory's facilities many projects can be assisted where large amounts of natural material must be produced and processed.

Proposed Course: To continue assisting NIH investigators when large-scale processing of biological materials is needed in their projects.

Publications: None

Serial No. NIAMD-LNE-3
1. Nutrition and Endocrinology
2. Vitamin Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in experimental nutrition

Previous Serial No. Same

Principal Investigators: Dr. F. S. Doft, Consultant, OD
Mr. E. G. McDaniel, LNE, VM

Other Investigators: None

Cooperating Units: Dr. G. Laqueur, M. Spatz (LEP-NIAMD)
Dr. R. L. Vought (EFS-NIAMD
Dr. G. Bieri and Dr. W. Rogers (LNE-NIAMD)

Man Years:
Total: 3
Professional: 1
Other: 2

Project Description:

Objectives: To determine the nutritional, biochemical and physiological characteristics of germfree and conventional laboratory animals.

Methods Employed: Germfree rats and mice are maintained on diets of known composition in sterile isolators. Then growth, blood picture, and other physiological functions are studied. Germfree animals are inoculated with known strains of bacterium to determine the organisms responsible for various phenomena. Conventional animals are used both in conjunction with germfree work.

Major Findings: The studies of the effect of cycasin, in collaboration with Drs. Laqueur and Spatz resulted in finding that cycasin was not toxic for germfree rats. When intestinal organisms supply the proper-β-glucosidase cycasin is as toxic as when fed to conventional animals.

This study is being continued to demonstrate the influence of various types of bacteria in causing the tumors which occur several months after single doses of cycasin are given. An experiment is also in progress to study the occurance of spontaneous tumors in germfree and conventional rats, which were observed in previous experiments, but were thought to occur with aging and to be unrelated to the administration of cycasin.

Experiments with Dr. Vought have shown that iodine metabolism is influenced by the bacteria of the intestinal tract. This work is being continued to study the nature of this influence and its effect upon the development of goiter.

With Dr. Bieri and Dr. Rogers a study of vitamin A deficiency in germfree, ex-germfree and conventional rats is in progress. Initial results indicate that the early death in vitamin A deficiency may be due largely to the inability of the animal to combat infection. Use of germfree animals is making it possible for the first time to study the direct effect of a severe vitamin A deficiency.

Dr. Doft in collaboration with Dr. D. B. Jelliffe (Director of the Caribbean Food and Nutrition Institute) is planning a series of experiments to study the possibility of controlling the intestinal flora in humans. It has been observed that laboratory animals on limited protein intake must compete with the intestinal bacteria for amino acids. It was observed that the effects of this competition be lessened if the intestinal flora could be reduced or controlled. It is hoped to reduce or control the intestinal flora of infants in a way to permit more efficient utilization of their normally inadequate diet. The preliminary experiments are being done with rats in this laboratory.

Significance to Bio-medical Research and the Program of the Institute. The work with the germfree animals will give us a better understanding of the relation between the animal's intestinal flora and its physiological reactions both to dietary nutrients and to other factors. The work with the germfree animals emphasizes the importance of nutrition in long-term studies.

Proposed Course of Project: Experiments will be conducted in an attempt to determine more precisely the relationship between the host animal and its bacterial flora with respect to its influence upon the nutrition and physiology of the host. Attempts will be made to identify the specific organisms which are responsible for such influence and, if possible, determine methods to counteract such influences as may be deleterious to the host.

Publications: None

Serial No. NIAMD-LNE-4
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of protein hormones: Chemistry and physiological actions of protein hormones.

Previous Serial Number: NIAMD-LNE-4

Principal Investigator: Dr. Robert W. Bates

Other Investigators: None

Cooperating Units: None

Man Years

Total:	3
Professional:	1
Other:	2

Project Description:

Objectives: To isolate protein hormones from pituitary glands of man and animals, functional, transplantable mouse or rat pituitary tumors, and blood, especially the anterior pituitary hormones; to investigate the nature of these hormones as they circulate in the bloodstream; to correlate chemical structure and biological function; to study the effect of hormones produced in excess by tumors on the host animal; to investigate the induction of diabetes by hormones.

Methods Employed: Hormones and other substances are extracted from pituitary gland, rat or mouse pituitary tumors, pancreas and blood. Crude extracts which may contain several hormones are fractionated by techniques commonly used in protein chemistry, such as salt precipitation, solvent fractionation, iso-electric precipitation, preparative and disc electrophoresis, ion-exchange chromatography, counter-current distribution, and gel filtration. Various quantitative biological assays are required to determine the concentration of the several hormones in tissues and protein fractions. Radioimmunoassay techniques are employed to extend the studies to concentration levels beyond the reach of conventional bioassay procedures. Removal of various endocrine glands with and without hormonal replacement is a critical technique. Effects of elimination or modification of hormonal production of the thyroid by anti-thyroid drugs and of the adrenal cortex by anti-adrenal drugs is compared effects of surgical removal of the gland.

Major Findings: Radioimmunoassay (RIA) of Insulin: In connection with our studies on induction of diabetes we have made a limited number of determinations of insulin concentrations in plasma and pancreas using the double antibody method of RIA. This double antibody method takes several days and requires a second antiserum, which is expensive and difficult to procure. Hence, for the past few months much time has been spent perfecting Dr. Kevin Catt's solid phase RIA which eliminates the second antibody reaction. We have found that plastic disposable microtiter plates which are used in immunology and contain 96 wells, are excellent for this purpose. The wells have a volume of 0.2 ml. These may be coated with the insulin antibodies by flooding the plate with anti-insulin serum. The plate is then washed several times to remove unbound anti-insulin serum. The test solutions and labelled insulin are then added and the plate is placed in a refrigerator overnight. In the morning the plate is rinsed with tap water several times to remove unbound insulin. The plate is then easily cut apart with scissors and the individual wells are placed in the usual tubes for counting of the bound radioactive insulin. The assay is completed in 24 hours. Satisfactory results can be obtained the same day with only 3 or 4 hours of incubation at room temperature but overnight incubation in a refrigerator gives more accurate results. The cost of materials and reagents for each assay is reduced to 20% and the labor by more than 50%.

Induction of Diabetes with Exogenous Hormones: Growth hormone and ACTH act synergistically to produce diabetes in partially pancreatectomized rats. When ACTH was replaced by various corticoids, hydrocortisone was found not to be synergistic with GH, whereas dexamethasone and corticosterone showed partial synergism.

Prophylaxis of Diabetes Induced by Endogenous Hormones: (1) Prophylactic Effect of Insulin. Every partially (80%) pancreatectomized (P) rat that bears the transplantable pituitary tumor MtT-F4, which makes GH and ACTH, will develop diabetes within one week. Such rats were treated daily for 21 days with an amount of insulin that prevented or permitted minimal glucosuria. This treatment prevented development of permanent diabetes in about three-fourths of the rats.

(2) Prophylactic Effect of Anti-Adrenal Drugs: Recently the drugs Metopirone and Elipten have been shown to interfere rather completely with certain pathways in the synthesis of adrenal steroids and thus reduce the levels of adrenal steroids in the body. Since adrenalectomy will prevent development of diabetes in about half of the 80% $\bar{P}$ rats with MtT, studies were made of the potentiality of Metopirone and Elipten as prophylactic agents for the prevention of diabetes. When 0.5% Metopirone or 0.1% Elipten were added to the diet of 80% $\bar{P}$ rats with MtT, the onset of diabetes was delayed for one to two weeks, but by 21 days the hormonal stimulus was so great that about half of the rats became severely, if not permanently, diabetic. When both drugs were added to the diet in the same concentration the protection was the same, about 50%. At the end of the 21 day treatment the concentration of corticosterone in plasma and adrenals were reduced to minimal normal control levels by Elipten and by the mixture. The total amount of corticosterone in

the adrenal, however, was still roughly normal because of the 3 to 6 fold enlargement of the adrenal. Elipten and Metopirone had a demonstrable prophylactic effect in reducing the onset and extent of development of diabetes, presumably by reducing adrenal steroid production by the ACTH produced by the tumor.

(3) Effect of Elipten and Metapirone on Splanchnomegaly: Addition of the anti-adrenal drugs to the diet permitted the enlargement of the liver, kidneys and heart which occurs in MtT tumor bearing rats. This is in contrast to the effect of adrenalectomy which has previously been shown to prevent completely splanchnomegaly. This new finding suggests again that some unrecognized product of the adrenal cortex is required for splanchnomegaly. Perhaps these are 18-OH corticoids, on the basis of the paper by Francois, Bates and Johnson.

Significance to Bio-medical Research and the Program of the Institute: One of the major roles of the six hormones or the anterior pituitary gland is to modify or regulate the size and functional activity of other glands or tissues, which in turn control the overall metabolic state of the individual. This hormonal balance or imbalance controls how one feels and what one can do. Certain metabolic abnormalities and tumors are corrected by treatment with hormones or by removal of endocrine glands. Purified hormone preparations are needed for this work together with sensitive and quantitative tests which will estimate the concentration of hormones in the blood or transport system and the rate of release of the hormones. One needs to know what factors control the biosynthesis and subsequent release of hormones by the pituitary gland. More knowledge concerning the hormonal induction of diabetes may be highly important.

Proposed Course: Studies on the induction of diabetes by hormones in rats that are 80% pancreatectomized will be carried out in a systematic manner to throw light on the problem of hormonal interactions (potentiation or inhibition) and to assess the significance of hormonal balance.

Analysis of hormone levels in rats and mice with transplantable pituitary tumors will continue. A search for factors which modify these levels will be made. The physiological action of the hormones from the pituitary tumors upon the host will be studied. Studies on the changes occurring after tumor removal are planned.

The question of identity of human GH and prolactin results in confusion when one attempts to establish potency of prolactin in plasma of humans in various clinical conditions. Also the specificity of the cropsac response in pigeons raises the question of whether the response obtained with urine and plasma extracts is due to prolactin. These problems are being investigated.

Honors and Awards: None

Publications:

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Epstein, C. J., Moses, H. L., Epstein, L. B. and Garrison, M. M.: A structural analysis of hepatomegaly induced by a hormone-secreting tumor. Experimental and Molecular Pathology 7: 304-326, Dec. 1967.

Francois, D., Bates, R. W. and Johnson, D. F.: In vitro metabolism of 4-^{14}C labelled Progesterone by adrenal glands from rats with a transplantable mammotropic pituitary tumor. Endocrinology 81: 246-250, Aug. 1967.

Macchia, V., Bates, R. W., and Pastan, I.: The purification and properties of a thyroid-stimulating factor isolated from Clostridum Perfringens. J. Biol. Chem. 242: 3726-3730, Aug. 1967.

Odell, W. D., Reichert, L. and Bates, R. W.: Pitfalls in the radioimmunoassay of carbohydrate containing polypeptide hormones. In Margoulies, M. (Ed.): Protein and Polypeptide Hormones. Amsterdam, Excerpta Medica Foundation, 1968, pp. 124-128.

Serial No. NIAMD-LNE-5
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Diabetes and Fat Metabolism

Previous Serial Number: NIAMD LNE-5

Principal Investigators: Drs. Robert O. Scow and Sidney S. Chernick

Other Investigators: Drs. Fredrick A. Bieberdorf, Charles M. Clark, Jr.,
Dr. Margit Hamosh, Visiting Scientist
Dr. Aubie Angel, Guest Worker
Drs. Yechezkiel and Olga Stein, Hebrew University
Hadassah Medical School, Jerusalem, Israel.

Cooperating Units: Departments of Medicine and of Experimental Medicine and Cancer Research, Hebrew University, Hadassah Medical School, Jerusalem, Israel.

Man Years:

Total:	9
Professional:	5
Others:	4

Project Description:

Objectives: To determine influence of hormones and other factors on metabolism of fat and carbohydrate in liver and adipose tissue of normal and diabetic animals.

Methods Employed: Animals are deprived of one or several endocrine glands and treated with various hormones or other substances. The effect of the above on metabolism of fat and carbohydrate in liver, adipose tissue and other organs and on blood hormone levels are determined with *in vivo* and *in vitro* studies using conventional and radioisotope techniques. Effects of hormones added *in vitro* are also studied.

Major Findings: Ketosis of Fasting: The effect of insulin on free fatty acid (FFA) turnover (using ^{3}H-oleic acid) and ketosis in the fasting rat was studied in vivo using a technique which allows continuous blood sampling for two hours. Blood loss is replaced by continuous transfusion. Acute insulin deprivation, induced by D-mannoheptulose and guinea pig anti-insulin serum, increased plasma FFA concentration, FFA production and FFA uptake, and at the same time caused the development of ketosis. Injection of insulin, in both large and small doses, rapidly decreased FFA production, lowered plasma FFA concentration and simultaneously abolished ketosis of fasting. No effect on ketosis could be obtained without a concurrent effect on FFA production. Studies thus far suggest that the effect of insulin on ketosis is mediated through its effect on FFA production and plasma FFA level. Studies of the effect of insulin on ketone body production and utilization in vivo are currently underway.

Hormonal and other factors that affect ketone body production are also being studied in the perfused isolated rat liver. It has been found that hepatic ketone body output is closely dependent on plasma FFA concentration and that insulin has no effect in the liver on ketone body production. These findings complement those made in the intact rat.

Studies on ketosis of fasting in pregnant rats have shown that the level of insulin in plasma is not low enough to explain increased lipid mobilization and hyperketonemia found in these rats. The possibility that a relative resistance to insulin exists in pregnant rats was investigated. Insulin administration (1.0 or 0.2 units) lowered the plasma concentrations of glucose, free fatty acids (FFA), ketone bodies and triglycerides in fasted pregnant and control rats. The plasma FFA concentration in the pregnant rats, which is higher than that in non-pregnant rats, did not fall to the low values seen in the control rats, even at 3 hours after insulin. This finding suggested that insulin may not completely suppress lipolysis in adipose tissue of fasting pregnant rats. In vitro experiments confirmed that fatty acid release in adipose tissue from fasted pregnant rats was greater than that from fasted controls and that it was not decreased by insulin injection. The data suggest that the lipolytic mechanism in adipose tissue is resistant to insulin during pregnancy. Since growth hormone and glucocorticoids are both needed for development of ketosis in the pregnant rat, it is possible that they are responsible for the insulin resistance seen.

During the course of the above studies, a new micro-procedure for measuring acyl glycerol was developed. It involves hydrolysis of acyl esters with tetraethanol ammonium hydroxide, which leaves phosphate esters intact, and the liberated glycerol is assayed enzymatically in a fluorimeter.

Studies of Ketosis in Hypophysectomized-Pancreatectomized Rats: Earlier studies showed that dexamethasone caused very severe ketosis in fasting hypophysectomized-pancreatectomized (HP) rats when the hormone was given one hour after hypophysectomy but not when given seven days later. It was then postulated that the ketogenic action of the glucocorticoid might require the presence of a hypophyseal factor which disappeared from the animal during the first week after hypophysectomy. Recently it was found that dexamethasone caused severe ketosis in HP rats deprived of the pituitary gland for one week if either growth hormone or ACTH Gel was given with the glucocorticoid; neither pituitary hormone was ketogenic when given singly. Dexamethasone also caused ketosis if the animals were pretreated 48 and 24 hours before with growth hormone. These findings indicate that glucocorticoids are ketogenic in HP rats but only if the animals are conditioned by either growth hormone or ACTH. The nature of this phenomenon is under study.

Effect of insulin on chylomicron uptake by perfused adipose tissue: Previous studies in this laboratory showed that during the removal of chylomicron glycerides from blood by perfused adipose tissue, about 3/4 of that removed was hydrolized to glycerol and free fatty acids and 1/4 was transferred intact to the tissue. The studies also showed that 70% of the fatty acids released from chylomicron glycerides, by the action of lipoprotein lipase, were incorporated into tissue glycerides. The effect of fasting and insulin lack on chylomicron uptake has now been studied. It was found that the amount of fatty acids split off from chylomicrons and incorporated into tissue lipids was 50% less in perfused fat pads of pancreatectomized and fasted rats than in those of fed rats. It was also observed that infusion of insulin, at a concentration 100 μU/ml blood, restored immediately this process to normal in perfused adipose tissue of pancreatectomized rats. Since preliminary results suggest that hydrolysis of chylomicron glycerides is rate limiting, these findings indicate that insulin may have a direct and immediate effect on lipoprotein lipase activity in adipose tissue. The site and nature of this action is now under study.

Fatty acid metabolism in perfused adipose tissue: Free fatty acids (FFA) are removed from blood, esterified and stored by adipose tissue as triglyceride. Biochemical studies have shown that the esterification process occurs in the particulate fraction and that there might be several pools of fatty acids in the tissue. It has been suggested that newly synthesized glyceride is transferred from site of synthesis to site of storage in the form of membrane-enclosed lipid droplets. The mechanism of FFA transfer from blood to fat cell is not known.

These processes were studied with electron microscope radioautography in perfused adipose tissue taken from starved-refed rats. The fat pads were perfused with diluted blood containing insulin (2 mU/ml) and glucose. The tissues were infused intraarterially with ^{3}H-oleic acid (complexed with albumin) for various times, ranging from 0.3 to 5.3 minutes, and then fixed immediately with cold glutaraldehyde which stopped at once all metabolic processes. Later, part of the tissue was used for chemical analyses and part for electron microscopy.

Since the vascular bed was not flushed of radioactive oleic acid, about 1/3 of the radioactivity in the tissue was present as FFA, or unesterified fatty acids, and the rest was present as esterified fatty acids. The labeled free fatty acids in the tissue were found associated with albumin. Whether the albumin-FFA complex was intravascular or extravascular was not determined, but it seems unlikely that it would be present within the fat cells.

More than 3/4 of the newly esterified fatty acid, even at 18 seconds, was in the triglyceride fraction and about 1/4 in diglyceride; the phospholipid fraction contained less than 1%. The rate of esterification of oleic acid was between 0.1 and 0.2 μEq/(min x g).

The radioautographic reaction, as determined with the electron microscope, was seen at all times predominantly over fat cells and rarely over connective tissue or endothelial cells. The silver grains in fat cells were seen mostly over lipid droplets of all sizes, much less frequently associated with endoplasmic reticulum and membrane-bound vesicles, and seldom with mitochondria. The relatively large size of silver granules, however, made localization of radiation source to specific intracellular organelles very difficult. No evidence was seen that triglyceride is transported in small lipid droplets.

The findings indicate that free fatty acids are rapidly transferred from the blood stream to the site of glyceride synthesis within the fat cell and the newly synthesized glycerides are then transferred directly to the nearest lipid droplet. The manner in which FFA and newly synthesized glycerides are transported within adipose tissue remains unknown.

Significance to Bio-medical Research and the Program of the Institute: The importance of the pituitary and adrenal glands in development of classical diabetes in pancreatectomized animals was first shown by Houssay and by Long and Lukens. Recent studies in this laboratory have shown that severe diabetic ketosis will develop in insulin deficient rat when glucocorticoids are given with either growth hormone or ACTH. The role of the pituitary hormones in this phenomenon is not known. These hormones are also needed for development of ketosis in fasting pregnant rats. The findings reported above suggest that resistance to insulin rather than a lack of circulating insulin is one of the causative factors. The studies of R. W. Bates in our laboratory have demonstrated that both growth hormone and ACTH are needed in excess to produce diabetes (glycosuria) in partially pancreatectomized rats; glucocorticoids, for some unknown reason, do not replace fully ACTH in this process. Diabetes is preceeded for several days by high plasma insulin levels.

As noted in previous reports, these observations indicate that diabetes and diabetic-like disturbances, such as ketosis and hyperlipemia, can occur in the presence of appreciable amounts of circulating insulin, but only if growth hormone and glucocorticoids, or ACTH, are present in excess amounts. The tissues in which these effects occur have not been identified; adipose tissue may be one. Once this has been done the intracellular sites of action should be looked for. The mechanism of interaction of these hormones with insulin is being studied.

Hyperlipemia in untreated diabetic and in fasted pregnant animals is corrected by insulin administration. The current studies on chylomicron uptake in perfused adipose tissue indicate that the effect of insulin on blood glyceride is probably secondary to its stimulatory effect on lipoprotein lipase, resulting in an accelerated removal of lipid from the blood. Another effect, of course, could be decreased mobilization of FFA. Lipoprotein lipase is located in many tissues of the body and has an important role in uptake of glyceride fatty acids by these tissues. Recent studies by Robinson in Oxford showed that the enzyme activity is decreased in adipose tissue during the last part of pregnancy and the early post-partum period in the rat, perhaps to divert blood glycerides to the fetuses and, after parturition, to the mammary tissue. In support of this, the enzyme activity in mammary tissue is increased immediately after delivery in the guinea pig and remains high as long as the mother suckles. These observations would suggest that another hormone, probably prolactin, affects selectively this enzyme in several tissues. The nature of the hormonal effects on this very important ubiquitous enzyme deserves intensive study.

The radioautographic studies with the electron microscope of fatty acid uptake and esterification by adipose tissue demonstrated that these are very rapid processes. Current concepts concerning intracellular glyceride transport and fatty acid pools in adipose tissue were not substantiated by the findings. Perhaps these discrepancies are due to technical problems. None the less, they need to be clarified. The results suggest that FFA transport between blood and fat cells occurs so rapidly that study of this phenomenon will require development of new research techniques. This transport process is important to study because it occurs in many tissues that have an unfenestrated capillary bed, namely muscle, heart, mammary gland, uterus, and adipose tissue.

Proposed Course: Study of hormonal factors involved in the development of increased lipid mobilization and ketosis will be continued. An attempt will be made to determine the site of action of these hormonal factors and the sequence of events that lead to ketosis and accumulation of triglyceride in blood and liver.

Studies of the effect of hormones and other factors on lipoprotein lipase in adipose tissue will be continued. The site of formation and action of the enzyme, as well as mechanism of action, and the role of the capillary endothelium in the hydrolysis of blood glycerides will be investigated.

An electron microscope is being installed this year in the laboratory. It will be used to investigate the effects of hormones on structure and function in adipose tissue, liver, blood capillaries and other tissues. It will also be used to localize hormones and enzymes within the cells. The role of the capillary endothelium in transfer of hormones, metabolic percursors and products, and enzymes between the blood stream and tissue cells will be studied.

Honors and Awards: None

Publications:

Scow, R. O., Stein, Y. and Stein, O.: Incorporation of dietary lecithin and lysolecithin into lymph chylomicrons in the rat. J. Biol. Chem. 242 No. 21, 4919-4924, Nov. 1967.

Scow, R. O.: Transport of triglyceride, its removal from blood circulation and uptake by tissues. In Meng, H. C. and Lse, D. H. (Eds.) Proceedings of an International Symposium on Parenteral Nutrition, Charles C. Thomas, 1968, (in press).

Serial No. NIAMD-LNE-6
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of Action of Hormones on Isolated Fat Cells.

Previous Serial Number: NIAMD LNE-6

Principal Investigator: Dr. Martin Rodbell

Other Investigators: Dr. Lutz Birnbaumer, Visiting Fellow
University of Buenos Aires, Argentina

Cooperating Units: None

Man Years

Total: 3
Professional: 2
Others: 1

Project Description:

Objectives: To determine the mechanism of action of insulin and lipolytic hormones (catecholamines, ACTH, glucagon) on isolated fat cells.

Methods Employed: Fat cells are isolated from adipose tissue of rats after dispersing the tissue with collagenase. Isolated fat cells are lysed by gently agitating fat cells in a hypotonic medium. This procedure results in the formation of sacs of plasma membrane, termed "ghosts", which are used for investigating the effects of hormones on glucose transport and metabolism as well as on adenyl cyclase. Adenyl cyclase activity is assayed using a new procedure which involves isolating labeled cyclic 3'5' AMP (cAMP) formed during hydrolysis of ATP-^{32}P by the enzyme. Conventional isotopic and chemical methods are employed in other studies of hormone action.

Major Findings: It was previously reported that the lipolytic hormones ACTH, epinephrine, and glucagon stimulate adenyl cyclase activity of fat cell "ghosts". A study of the hormone--adenyl cyclase interaction was undertaken to explore whether one or more enzymes are involved and to obtain some information on the primary site of action of these hormones and perhaps on their primary mechanism of action. It was found that the stimulatory effects of the three hormones when tested together, were not additive when maximally stimulating concentrations were used. It was concluded that only one enzyme is affected by them.

Propranolol, an inhibitor of the β adrenergic effect of epinephrine, inhibited only the stimulation obtained with the catecholamine. The activation achieved with the polypeptide hormones, ACTH and glucagon, was not affected by the β blocker. Combination of the hormones in sets of two at half concentrations caused a higher activation of adenyl cyclase than when the single hormones were added near their K_a. These experiments suggest that each hormone acts via a specific receptor site, that adenyl cyclase is not the primary site of action of these hormones, and that hormone specificity is determined by receptor sites present in the outer plasma membrane. The K_a found for ACTH, glucagon, and epinephrine are 3.0×10^{-7}M, 0.9×10^{-7}M, and 60×10^{-7}M, respectively. The optimal pH for enzyme activity as well as for hormone stimulation (determined with ACTH) is 8.2. ATP concentration curves at constant Mg:ATP ratios are sigmoid in shape. Addition of ACTH accentuates the sigmoidal shape, confirming the idea that the hormones act allosterically on adenyl cyclase activity.

Significance to Bio-medical Research and the Program of the Institute: The finding that sacs of plasma membrane isolated from fat cells retain the hormone responsiveness shown with intact cells indicates that this material provides a new tool for investigating the mechanisms of actions of hormones. The evidence suggests that the plasma membrane is the site of action of insulin and lipolytic hormones. Adenyl cyclase is a key enzyme in the action of lipolytic hormones in fat cells and can be used as a marker for further purification of the plasma membrane of the fat cell.

Proposed Course: Continued efforts will be made to purify the plasma membrane of "ghosts" and to correlate hormone action with the structure and function of this membrane. A detailed study of the properties of adenyl cyclase and the action of lipolytic hormones and insulin on this material has been initiated. Studies of the action of insulin on carbohydrate, amino acid, and ion transport in "ghosts", are now in progress.

Honors and Awards: None

Publications:

Rodbell, M.: Metabolism of Isolated Fat Cells. V. Preparation of "ghosts" and their properties; adenyl cyclase and other enzymes. J. Biol. Chem. 242: No. 24, 5744-5750, Dec. 1967.

Rodbell, M.: Metabolism of Isolated Fat Cells. VI. The effects of insulin, lipolytic hormones, and theophylline on glucose transport and metabolism in "ghosts". J. Biol. Chem. 242: No. 24, 5751-5756, Dec. 1967.

Serial No. NIAMD-LNE-7
1. Nutrition and Endocrinology
2. Nutritional Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Biochemical studies related to nutrition.
Previous Serial Number:	NIAMD-LNE-7
Principal Investigators:	Drs. J. G. Bieri, J. N. Williams, Jr. and H. G. Windmueller
Other Investigators:	Drs. M. E. Reid and W. E. Rogers, Jr.; Mrs. E. L. Prival, Mr. A. E. Spaeth and Mrs. S. L. Thorp
Cooperating Units:	Dr. Robert I. Levy, LM, NHI Mr. E. G. McDaniel, LNE, NIAMD Dr. K. E. Mason, EP, NIAMD
Man Years	
Total:	8
Professional:	5
Other:	3

Project Description:

Objectives. To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed. Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

The relationship of nutrition to other scientific areas, such as neurology and pathology, is studied.

Major Findings.

A. Metabolism and Function of Fat Soluble Vitamins and Related Substances. (J. G. Bieri and W. E. Rogers, Jr.)

Studies of the systemic biochemical function of vitamin A have been directed toward specific enzymes and also toward observations of the whole animal. The effect of vitamin A deficiency on the activities of enzymes revealed that L-gulono-γ-lactone oxidase, present in liver microsomes, was reduced to about one-tenth that in normal liver. In view of the known effects of food-intake on this enzyme, and the inanition that vitamin A deficient rats undergo, the significance of these factors is currently being evaluated. In a study to evaluate the contribution of infection to the total condition of the vitamin A deficient rat, growth and survival in the germ-free condition have been studied in collaboration with Mr. E. G. McDaniel (Section on Vitamin Metabolism). Whereas littermate rats removed from the isolator at weaning and maintained on a vitamin A deficient diet died in 5-6 weeks, those kept inside the isolator on the same diet have survived for five months. A few deaths in the germ-free rats were attributed to intestinal strangulation or to bladder stones. The vitamin A deficient, germ-free rats stopped growing in seven weeks at a weight of about 200 grams and developed a hind leg weakness not seen in the conventional rats. One germ-free deficient rat when killed after four months had very small testes and corneal and retinal lesions. This study so far has shown that infection is the cause of death in the conventional vitamin A deficient rat, and that the vitamin, although essential for certain specialized tissues, is not critical for the survival of the animal in the absence of infection.

In continuing studies of the vitamin E activity and metabolism of N-substituted tocopheramines, the biopotency of dl,N-methyl-β-tocopheramine was determined in the rat gestation-resorption assay. It was found to have 90% of the activity of dl,α-tocopherol, thus substantiating its full vitamin E potency found in our previous assays with the chick. The metabolism in the rat of N,N-dimethyl-γ-tocopheramine, reported by Dam's group to be active in the chick, was investigated. It was found that this compound was demethylated to N-methyl-γ-tocopheramine. Since we had shown previously that this compound has high vitamin E activity, this would explain the activity of the dimethyl compound which on theoretical grounds should be inactive.

B. Biochemical Studies of Cytochromes. (J. N. Williams, Jr. Work done in laboratories of Dr. E. C. Slater, University of Amsterdam.)

Existing procedures for isolation of cytochrome b give a highly degraded product with no enzmatic activity. In beef heart mitochrondrial extracts, cytochromes b and c_1 are present in a complex which when split with 5% cholate - 25% saturated ammonium sulfate gives an insoluble cytochrome b and a soluble cytochrome c_1. Such cytochrome b is impure and almost completely reactive with carbon monoxide (indicating complete denaturation). We undertook further purification of this insoluble product by treating it with a bacterial protease, Nagarse. The cytochrome b became soluble in Tween 20 - phosphate buffer but was still almost completely reactive with carbon monoxide. It had 32 μmoles of b per gram of protein, indicating very high purity. Properties of this preparation were: reaction with NADH anaerobically if a trace amount of b-c_1 complex was added, with a ratio of NADH: cytochrome b of 8:1; reduction (about 20%) in the presence of succinate and catalytic amounts of b-c_1 complex.

In a second purification attempt, the b-c_1 complex was applied for a short time to the upper part of a Sephadex G-100 column and then rapidly eluted. The complex split and cytochromes b and c_1 were partially separated. This cytochrome b was only 20-30% reactive with carbon monoxide. Precipitation with ammonium sulfate increased CO-reactivity to 45-50%. In other tests of purity, the preparation was reduced by NADH almost stoichiometrically with either phenazine methosulfate or a catalytic amount of unsplit b-c_1, and was reduced with succinate to about 40%. These indicate considerably greater purification than by conventional procedures. The best preparation of cytochrome b obtained by splitting on a Sephadex column was estimated to be 50% pure.

C. Lipid Synthesis and Transport. (H. G. Windmueller)

We have developed a simple and reliable procedure for collecting large quantities of lymph directly from the intestinal mesentery of the rat which has permitted analyses of lipids and lipoproteins produced by the intestine. It has been found that intestine produces β-lipoprotein and triglycerides even when the animal is on a fat-free diet. In normal rats on a fat-free diet, intestinal lymph contained higher concentrations of triglycerides and β-lipoprotein and lower concentrations of α-lipoprotein than did plasma. In rats fed orotic acid, intestinal lymph contained as much triglyceride and β-lipoprotein as in controls, although the plasma concentrations of both were less than 5% of normal. We have shown previously that orotic acid feeding totally blocks β-lipoprotein production by the liver as well as triglyceride release from the liver into plasma. Together these findings indicate that intestine as well as liver normally releases β-lipoprotein into the circulation, and that in both organs β-lipoprotein is associated with the efflux of cellular triglyceride.

It had been generally assumed that the liver was the only source of plasma triglyceride when animals are on a fat-free diet. Lymph analyses now reveal that about 10% of endogenous plasma triglyceride is derived from the intestinal mucosa and reaches the blood via lymph in association with β-lipoprotein. Studies in rats with tritium-labeled body water indicated, however, that the fatty acids of this intestinal triglyceride were not newly synthesized de novo but that they reach the intestinal mucosa as phospholipid in either bile or plasma.

On a fat-free diet, the triglyceride and β-lipoprotein of lymph were found in a group of particles all of density less than 1.006 g/ml and having the size and ultracentrifugal flotation characteristics of chylomicrons. Such large particles have been thought to be associated only with lymph transport of exogenous (dietary) fat. When fat was fed, the same range of lymph lipoprotein particles was found, only the proportion of chylomicrons was higher. We have therefore proposed that a β-lipoprotein-containing complex is the transport vehicle for endogenous as well as exogenous triglyceride from the intestinal mucosa into lymph, and that the size, flotation rate and lipid composition of the complex varies with the amount of fat transported.

Significance to Bio-medical Research and the Program of the Institute. A more complete understanding of the nutrition, biochemistry and metabolism of essential amino acids, proteins, vitamins, minerals and fatty acids in different living organisms can be expected to contribute still further to our knowledge of the roles of these essential nutrients in human beings. It is well established that the nutrition of man plays a role in the etiology of many degenerative and metabolic diseases, certain infectious and neurological diseases and some types of cancer. Basic studies in nutrition and biochemistry of nutrients may provide the means to prevent or cure some of these diseases.

Proposed Course of Project. Clarification of nutritional responses and explanation of interrelationships at the biochemical level remain the primary goals of the project.

Publications:

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Bieri, J. G.: Chromatography of tocopherols. In Marinetti, G. (Ed.): Lipid Chromatographic Analysis. New York, N. Y., Marcel Dekker, Inc., 1968, Volume 2, in press.

Reid, M. E. and Bieri, J. G.: Nutritional studies with the guinea pig. VIII. Thiamine. Proc. Soc. Exp. Biol. Med. 126: 11-13, Oct. 1967.

Rogers, W. E., Jr., and Bieri, J. G.: Δ^5-3β-hydroxysteroid dehydrogenase and vitamin A. J. Biol. Chem. June 1968, in press.

von Euler, L. H. and Windmueller, H. G.: Fatty liver in the rat after intravenous infusion of orotic acid. Proc. Soc. Exp. Biol. Med. 125: 1251-1254, Aug. 1967.

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ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Carbohydrate Metabolism

The DPN-dependent cyclization of glucose-6-P to inositol-1-P has been studied with a partially purified glucose-6-P cyclase prepared from rat testis. In the presence of enzyme and NAD, the rate of inositol-1-P formation from specifically deuterated as well as unlabelled glucose-6-P was compared. A kinetic isotope effect was observed in the cyclization of glucose-6-P, 100% labelled with deuterium in positions 1, 5, and 6. Glucose-6-P,6d$_2$ inhibits the formation of inositol-1-P 63%; glucose-6-P,5d, 51%; and glucose-6-P,1d, 24%, compared to control glucose-6-P. From their magnitude the first two effects are probably primary isotope effects reflecting cleavage of C-H bonds at positions 5 and 6, consistent with a mechanism of cyclization of glucose--6-P involving oxidation at C-5 by NAD followed by formation of a C-C bond by an aldol condensation with release of H^+ at C-6, and reduction by NADH to inositol-1-P. The isotope effect seen with glucose-6-P,1d is higher than expected since no C-H bond cleavage should occur at C-1, although a secondary effect of D on the polarization of the aldehyde group inhibiting its tendency to form a carbonium ion might account for the effect.

Inositol isolated from the three experiments with labeled substrate was found to contain a single atom of D per molecule consistent with the mechanism postulated; i.e., loss of D at C-5 of glucose-6-P and replacement by NADd at C-2 of inositol-1-P; loss of D^+ at C-6 leaving 1D bound; and no involvement of D at C-1. (Dr. F. Eisenberg, Jr.)

An examination of the biochemical defects in the genetic disorders of mucopolysaccharide metabolism (Hurler, Hunter and Sanfilippo syndromes) has been undertaken utilizing fibroblast cultures obtained from skin biopsies of patients with the appropriate syndrome. The kinetics of sulfated mucopolysaccharide turnover in normal fibroblasts show that about 2/3 of the newly synthesized mucopolysaccharide (presumably, chondroitin sulfate B) is secreted into the medium while 1/3 is diverted to an intracellular pool where it is slowly degraded. Fibroblasts from Hurler and Hunter patients synthesize and secrete sulfated mucopolysaccharide at a normal rate; likewise, they fill the intracellular pool at a normal rate but fail to empty it because of inability to degrade the mucopolysaccharide.

Preliminary experiments have shown that mixing Hurler and Hunter fibroblasts causes a correction of the defect in both. The defect in Hurler fibroblasts is likewise corrected by the admixture of cells from Hunter, Sanfilippo and normal cultures, but not from another Hurler culture. These results, though incomplete, suggest that fibroblasts from each of the genetic forms lack a factor required to degrade mucopolysaccharides, which can be supplied (*via* the medium) by normal cells or by cells of the other two variants.

Implicit in these findings is the possibility that specific factors may be isolated to serve as replacement for those missing in the patients. (Drs. J. C. Fratantoni and E. F. Neufeld)

Attempts to ascertain the mechanism by which polysaccharides are enzymatically synthesized and assembled into the structure of a cell wall has been studied in yeast preparations. Here, the cell wall is known to contain two major carbohydrate components, mannan and glucan as well as a minor component, chitin, which is located in the bud scar region of the cell wall. It has now been found that a particulate preparation from yeast protoplasts catalyzes the incorporation of C^{14}-acetylglucosamine from UDP--acetylglucosamine into a material insoluble in 55% ethanol. The radioactive material has been provisionally identified as chitin by its insolubility in acid and alkali as well as by the release of acetylglucosamine after treatment with a chitinase and of glucosamine after acid hydrolysis. The enzymatic activity has an absolute requirement for Mg^{++} or Mn^{++} and is also activated about 5-fold by 0.04 M free acetylglucosamine. Glucose and glycerol at very high concentrations, but not acetylgalactosamine, can substitute for acetylglucosamine.

Protoplast lysate supernatants or whole cell extracts inhibit the activity of the chitin synthetase. At least part of the inhibitory power seems to reside in heat-stable, nondialyzable material. The inhibition is eliminated by treatment of the substance with alkali or with proteases, but not with ribonuclease. Thus, the inhibitor appears to be of proteinaceous nature and is being further investigated. (Drs. L. B. Rothman and E. Cabib)

Glycoprotein Studies

Ceruloplasmin. A technique was described in the last annual report whereby the plasma protein, ceruloplasmin, was modified and made radioactive by treatment with neuraminidase, galactose oxidase and sodium borotritide. Injection of this material into rabbits has now provided new insights which appear to be of basic importance for the understanding of the pathology of Wilson's disease (carried out in conjunction with Professor A. Morell, Albert Einstein College of Medicine, New York City).

Removal of one or more of the sialic acid residues on the various carbohydrate chains attached to the protein results in the exposure of a new, nonreducing sugar, D-galactose. In the presence of this terminal monosaccharide, the circulating protein is rapidly and quantitatively removed from the blood and accumulated within the parenchymal cells of the liver. The rapidity of this translocation appears to be uniquely dependent upon the presence of intact, terminal galactosyl residues; alteration or removal of this sugar obliterates the phenomenon.

Subsequent studies, directed towards evaluation of the role of the enzyme, sialyl transferase, in the etiology of the disease state, revealed a markedly lower enzyme titer in biopsy samples of livers from patients suffering from Wilson's disease. This finding, in turn, has been traced to the presence of an inhibitor, tentatively identified as an unsaturated fatty acid which accumulates in the liver. Current studies are being directed towards the metabolic lesion which causes the abnormal hepatic levels of free fatty acids. (Drs. J. Hickman and G. Ashwell)

Ribonuclease A and B. An alternate approach to a study of the role of protein-bound carbohydrates is being explored with the use of ribonuclease A and B as model compounds. The latter enzyme, B, is identical to A in all known parameters (activity, molecular weight, amino acid composition, etc.) with the exception that RNase B contains a carbohydrate chain consisting of 6 residues of D-mannose and 2 residues of N-acetyl-D-glucosamine.

Sequential enzymatic removal of these monosaccharide units, followed by extensive purification of the resulting protein species, has led to the isolation of a protein tentatively identified as RNase A. Efforts are currently underway to isolate and purify the linkage enzyme responsible for cleavage of the primary bond between N-acetylglucosamine and asparagine. (Drs. L. Van Lenten and G. Ashwell)

Antibody Synthesis and Secretion. While radioactive amino acids are incorporated into intracellular γ-globulin without delay, the initial extracellular appearance of such γ-globulin requires about 20 minutes. Similar results were obtained when the carbohydrate component of γ-globulin was selectively labelled with H^3-galactose and H^3-glucosamine. Thus, for γ-globulins labelled in their amino acid or carbohydrate component the curves for the initial appearance of intracellular and extracellular γ-globulin are parallel and separated by an interval of 20 minutes. These findings indicate that at least some of the galactose and glucosamine residues are acquired close to the time of synthesis of the polypeptide chains. In contrast, it is clear that the single sialic acid residue of γ-globulin is acquired close to the moment of secretion rather than synthesis because the curves for the intracellular and extracellular appearance of sialic-labelled γ-globulin are superimposable. When H^3-glucose was used to label the carbohydrate component, the labelled γ-globulin appeared extracellularly without delay. Since, in such experiments, the sialic acid was selectively removed prior to analysis, the absence of a secretory lag indicates that some sugars other than sialic acid are acquired relatively close to the time of secretion. These observations demonstrate that the carbohydrate component is slowly synthesized over the 20-minute period between the synthesis of the polypeptide chains of γ-globulin and the initial extracellular appearance of this protein.

The cell-free incorporation of C^{14}-galactose into a microsome associated, endogenous γ-globulin acceptor was demonstrated. Although the labelled γ-globulin sedimented with the microsomes, it could be solubilized from this structure by treatment with 0.01 N HCl, sonic oscillation or deoxycholate. The fact that greater than 80% of the solubilized, protein-linked, radioactivity was accounted for as γ-globulin indicates that the product was compartmentalized within the microsomes. Sonic treatment of microsomes prior to the incorporation assay resulted in complete inhibition of the formation of C^{14}-galactose labelled γ-globulin without inhibition of incorporation into other glycoproteins. Thus it would appear that there is a special mechanism for the synthesis of the carbohydrate component of γ-globulin and that this mechanism is related to the fact that γ-globulin is

the sole protein (glycoprotein) secreted by lymph node cells.
(Drs. R. M. Swenson, R. P. D'Amico and M. Kern)

Ribonucleic Acids

Studies on the Stability of Messenger RNA. Both *E. coli* RNase II and polynucleotide phosphorylase have been implicated as enzymes responsible for the breakdown of messenger RNA *in vivo* and *in vitro*. The question then arises as to what protects messenger RNA from degradation. It is partially protected when it is involved in a stable complex with ribosomes. At 37°, using poly U (synthetic messenger RNA) in a complex with ribosomes is readily degraded by RNase II. Stabilization of the complex by any of the following conditions leads to some protection; 1) decreased temperature, i.e., 10°, 2) addition of phenylalanine-transfer RNA to the complex, 3) fixation of the poly U to the ribosomes by treatment with formaldehyde (the poly U itself is thereby not modified but it is trapped onto the ribosome even at 10^{-4} M Mg^{++}). Current interpretation of these results is based on the extent of protection in each case and on the known properties of RNase II. The enzyme begins degrading at the free terminal C-3'-hydroxyl end of the poly U and continues until it meets a point of stable ribosome binding, when it stops. The remainder of the poly U is protected. Similar experimental results with polynucleotide phosphorylase suggest a similar interpretation.
(Drs. J. Castles and M. Singer)

Studies with Pure Nucleotide Phosphorylase. As isolated from *Micrococcus luteus* (formerly *M. lysodeikticus*) polynucleotide phosphorylase is homogeneous with respect to molecular weight (255,000 s_w^{20}, 92). In guanidine-HCl, in the presence of -SH, it breaks into subunits of molecular weight 64,000. On disk gel electrophoresis the pure enzyme shows at least six protein bands each of which coincides with a band of activity. These forms can be partly resolved by chromatography on hydroxylapatite and DEAE. The enzyme catalyzes polymerization with no requirement for oligonucleotide primer. It can be oxidized, under mild conditions, to give form that does require primer.

Another, and distinct primer-dependent form is obtainable by limited tryptic hydrolysis. This enzyme has a molecular weight of 220,000 (58,000 in guanidine-HCl) and an s_w^{20} of 9.0. Concomitant with tryptic hydrolysis all of the original electrophoretic bands are converted to a new, single band containing all the protein and activity. This form becomes primer independent again upon reduction with sulfhydryl compounds. Reduced enzyme can be reconverted to primer dependence by the common sulfhydryl inhibitors. These manipulations do not significantly affect the overall ability of the enzyme to catalyze polymer synthesis (in the presence of primer) or phosphorolytic degradation of polyribonucleotides.

The ability to initiate polymerization appears to be related to the nature of one or more sulfur residues on the protein. It is possible that during trypsin treatment these residues become easily oxidizable, yielding a primer-dependent form. The loss of a part of the polypeptide would then be only indirectly responsible for the functional modification. It is possible that the functional interconversions are indeed most directly influenced by conformational changes accompanying the chemical modifications. (Drs. C. Klee, R. Moses and M. Singer)

The Thionucleotide Components of Transfer RNA. The purification of the sulfurtransferase, which transforms tRNA uridine into 4-thiouridine, has been markedly improved by the introduction of a dextran partition step. Inclusion of this procedure, together with other improvements, offers promise of an overall purification of 2000-fold although this number is somewhat misleading because of simultaneous activation.

Further work on the state of the 4-thiouridine (4-TU) residues in tRNA, using urea or guanidinium chloride solutions as solvents during thermal denaturation, indicates that about one-third of the 4-TU in unfractionated tRNA is stacked rather than hydrogen bonded in the structure. Since almost all of the 4-TU occurs next to purine bases, this may represent a purine-4--TU stacking interaction. (Drs. J. Abrell, E. Kaufman and M. N. Lipsett)

Deoxyribonucleic Acids

Biochemical Studies of Lysogeny. Studies of the synthesis, structure and biological significance of the concatenated, circular and linear forms of λ phage DNA are continuing. Electron microscope studies with Dr. Paul Bartl have shown that the replicative forms of λ DNA include concatenated molecules 2-6 times the size of mature λ DNA. Circular λ DNA molecules, both supercoiled and open forms, are also important replicative intermediates. Circular structures 2-3 times the size of monomeric λ circles have been found and may be of significance in replication and/or recombination. (Drs. L. Salzman and A. Weissbach)

A New Phage-Directed DNase. A new DNase capable of attacking and destroying the twisted supercoil form of circular λ DNA has been found in λ infected cells. This enzyme is a phage-directed enzyme and has only been detected in thymine-requiring cells which are unable to synthesize DNA under thymineless conditions. The properties and genetic control of this enzyme are being studied. (Drs. R. Shuster and A. Weissbach)

DNA Polymerase. DNA polymerase from hela cells has been purified several-hundred-fold and its properties studied. The most significant finding is that this polymerase prefers single-stranded DNA as a template and is unable to use Vaccinia DNA as a template. Attempts are in progress to isolate a new DNA polymerase from Vaccinia-infected hela cells and study its properties. (Drs. K. Berns and A. Weissbach)

Enzymatic Utilization of Model Compounds in Bacterial Systems

These studies have continued a 2-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups and, as well, on those features of proteins responsible for specific catalysis. In the former category are the elucidation of pathways for the bacterial synthesis and degradation of tartaric acid (epoxysuccinate →

→tartrate → oxaloglycolate → + CO_2) (Dr. R. H. Allen and Dr. L. D. Kohn) and for maleic acid (maleate → fumarate → malate) (Dr. W. Scher). Contributions in the latter group involve preparative and analytical methodology as well as an analysis of structural and catalytic properties of aldehyde dehydrogenase (Dr. J. F. Clark).

The last of these projects, the properties of a yeast aldehyde dehydrogenase, is being studied in detail. During isolation the protein is extremely sensitive to proteolysis by enzymes present in the extracts and it has only been recently that preparations with a single amino acid end group have been obtained. Thus, although a crystalline and apparently homogeneous preparation was obtained two years ago, it is only now that what may be termed microheterogeneity has been eliminated. The active enzyme is a relatively large protein composed of an as yet undetermined number of monomeric units. The dissociation and reassociation of monomers is presently being analyzed in terms of kinetics of both changes in size and catalytic activity. (Dr. W. B. Jakoby)

Studies on Induced Permease Systems in E. coli

Permease accumulated pools of various substrates in *E. coli* have been found to be extremely sensitive to simple cold shock. Although cold shock does not impair either the viability of cells, nor their subsequent capacity to accumulate substrates, rapid temperature transitions from ambient to below 5° may result in the total loss of accumulated substrate within 15 seconds. The susceptibility of a pool to cold shock varies not only with the strain of *E. coli* and the osmotic composition of the medium, but with the nature of the carbohydrate used as carbon source during cell growth. It has been possible to demonstrate the reverse process; i.e., the rapid uptake of a labelled substrate under conditions of cold shock in the absence of a specified permease. These results suggest that the effect of cold shock may be to induce the formation of transient pores in the permeability barrier of the cell. (Dr. I. Leder)

Purification of Thiamine Phosphate Pyrophosphatase from Yeast

In yeast studies, the enzyme thiamine phosphate pyrophosphorylase has been purified to crystallinity from baker's yeast. It migrates as a single component in electrophoresis with a molecular weight exceeding 200,000. The synthesis of this enzyme is repressed by thiamine, but only limited feedback inhibition by thiamine, thiamine phosphate or thiamine pyrophosphate has been observed. Procedures for the synthesis of crystalline substrates for this enzyme have been devised. (Dr. I. Leder)

Hormone Dependent Differentiation of Mammary Gland in vitro

It has been established that the three hormones, insulin, hydrocortisone and prolactin, cannot elicit biochemical or morphological differentiation of *parent* mouse mammary epithelial cells. Rather, when epithelial cell division occurs in the presence of these hormones the *daughter* cells are differentiated. Partial resolution of this triple hormone system has been accomplished. Insulin is necessary and sufficient for the initiation of DNA synthesis in tissue derived from mature virgin and pregnant mice. Insulin is also required during the post-mitotic phase of the cell cycle. Hydrocortisone, in this system, must be present during the proliferative phases of the cell cycle. This is at least one of the reasons why the overall process is dependent on cell division. Prolactin can act post-mitotically. Thus, two types of mammary epithelial cells can be formed _in vitro_, neither of which is capable of synthesizing milk proteins. Cells formed in the presence of insulin alone cannot subsequently respond, in terms of milk-protein synthesis, to the addition of hydrocortisone and prolactin. Cells formed in the presence of insulin and hydrocortisone can respond to the post-mitotic addition of prolactin. The induction of casein synthesis by the post-mitotic action of prolactin appears to start from what approaches a zero-baseline synthetic capacity.

The initiation of epithelial cell DNA synthesis by insulin is due, at least in part, to the appearance of DNA polymerase activity. Androgens inhibit the overall differentiation process by inhibiting DNA synthesis by mammary epithelial cells.

Explants from the mammary glands of 3-week-old mice can be induced to synthesize casein _in vitro_ in the absence of lobuloalveolar development. Maximum biochemical differentiation requires the presence of insulin, hydrocortisone, and prolactin in the culture medium. In contrast to explants from adult mice, the mammary epithelium of immature animals undergoes DNA synthesis and mitosis _in vitro_ in the absence of exogenous insulin; however, such proliferation does not lead to the formation of differentiated daughter cells. Insulin acts in at least two ways during the proliferative phases of the cell cycle of differentiating mammary epithelial cells.

It was previously reported that both insulin and prolactin are required during the post-mitotic phases of the process. It now appears that, during this period, prolactin acts before insulin. (Drs. J. J. Elias, M. C. Green, E. S. Mills, E. W. Sutherland, A. E. Voytovich and Y. J. Topper)

Development of an Ultra-micro Assay for Glutathione

A method of glutathione assay has been developed which is based on the spectrophotometric measurement of the rate of color formation at 412 mμ in a mixture comprising TPNH, yeast glutathione reductase, the sulfhydryl reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), and the sample to be analyzed. The formation of color at the stated wavelength is due to reduction of essentially colorless Ellman's reagent to an intensely colored product in the presence of a sulfhydryl-containing compound such as reduced glutathione. A suitable control mixture is employed to correct for the rate of reduction of Ellman's reagent in the absence of added glutathione.

The enzymatic procedure outlined here is based on the observation that glutathione can act catalytically in the reduction of Ellman's reagent by TPNH in the presence of glutathione reductase, i.e., a very small amount of glutathione can catalyze the reduction of a very large amount of reagent.

The rate of this reduction depends in a strictly rectilinear manner on the amount of glutathione present in the mixture. Because of the catalytic role played by glutathione in this system the sensitivity of the procedure is 10 to 100 times that exhibited by previously published methods and is, as a consequence, suitable for the quantitative analysis of glutathione in tissue fluids (plasma, saliva, urine) normally containing very low levels (ca. 1 μg/ml) of the peptide. Furthermore, the method is unique in that it effectively measures total glutathione (i.e., GSH + GSSG) content. In addition, large excesses of other nonglutathione sulfhydryl compounds (e.g., cysteine) do not interfere. (Dr. F. Tietze)

Serial No. NIAMD-LBM-1
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biosynthesis of Inositol in the Mammal

Previous Serial Number: NIAMD-LBM-10

Principal Investigator: Dr. Frank Eisenberg, Jr.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 2-1/4
Professional: 2
Others: 1/4

Project Description:

Objectives - Elucidation of the mechanism of cyclization of glucose 6-P to inositol 1-P.

Methods Employed - A partially purified soluble enzyme, glucose 6-P cyclase, prepared from rat testis, was incubated with NAD and synthetic glucose 6-P, variously labeled with deuterium. The activity of the cyclase was measured in terms of synthesis of inositol 1-P from each substrate compared with the rate from unlabeled glucose 6-P. Inositol 1-P was assayed after enzymatic hydrolysis as inorganic phosphate photometrically and inositol hexatrimethylsilyl ether gas chromatographically. High resolution mass spectrometry was used to determine the amount of deuterium in the inositol ethers.

Major Findings - A kinetic isotope effect has been observed in the cyclization of glucose 6-P 100% labeled with deuterium in positions 1, 5, and 6. Glucose 6-P,6d_2 inhibits the formation of inositol 1-P 63%; glucose 6-P,5d, 51%; and glucose 6-P,1d, 24%, compared to control glucose 6-P. From their magnitude the first two effects are probably primary isotope effects reflecting cleavage of C-H bonds at positions 5 and 6, consistent with a mechanism of cyclization of glucose 6-P involving oxidation at C-5 by NAD followed by formation of a C-C bond by an aldol condensation with release of H^+ at C-6, and reduction by NADH to inositol 1-P. The isotope effect seen with glucose 6-P,1d is higher than expected since no C-H bond cleavage should occur at C-1, although a secondary effect of D on the polarization of the aldehyde group inhibiting its tendency to form a carbonium ion might account for the effect.

Inositol isolated from the three experiments with labeled substrate was found to contain a single atom of D per molecule consistent with the mechanism postulated; i.e., loss of D at C-5 of glucose 6-P and replacement by NADd at C2 of inositol 1-P; loss of D^+ at C-6 leaving 1D bound; and no involvement of D at C-1.

Significance to Bio-medical Research and the Program of the Institute - Delineation of a new pathway of carbohydrate metabolism in the male gonads is of interest. Pathology in this organ might be reflected in biochemical abnormalities in this pathway.

Proposed Course of Project - Kinetic isotope effects will be studied further with glucose 6-P labeled with D at C-3, the position most remote from the point of ring closure, in order to establish whether the inhibition observed with labels at C-5 and C-6 are authentic primary isotope effects on C-H splitting and not merely a reflection of an effect of the isotope on binding of substrate to enzyme.

NAD isolated from the enzymatic reaction mixture will be examined for incorporation of deuterium in order to assess its role in the cyclization.

Honors and Awards: None

Publications:

Eisenberg, F., Jr.: D-Myoinositol 1-phosphate as product of cyclization of glucose 6-phosphate and substrate for a specific phosphatase in rat testis. J. Biol. Chem. 242: 1375-1382, Apr. 1967.

Eisenberg, F., Jr. and Bolden, A. H.: Formate contamination in borohydride reduction. Carbohydrate Res. 5: 349-350, Nov. 1967.

Eisenberg, F., Jr.: Biosynthesis of inositol in the mammal. In Encyclopedia of Science and Technology, Rev., New York, McGraw-Hill, in press.

Serial No. NIAMD-LBM-2
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzymatic Reduction of Protein Disulfide Linkages in Mammalian Cells. (Subproject: Development of a New Enzymatic Procedure for the Quantitative Assay of Nanogram Amounts of Glutathione).

Previous Serial Number: NIAMD-LBM-8

Principal Investigator: Dr. Frank Tietze

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1-1/4
Professional: 1
Others: 1/4

Project Description:

Objectives - During the course of investigation of a disulfide-reducing enzyme system of rat liver (see preceding Annual Report) it became of interest to measure quantitatively the essentially trace levels of free glutathione remaining in thoroughly dialyzed preparations. Since published methods of glutathione assay were insufficiently sensitive for this specific purpose it seemed worthwhile, as a subproject, to investigate modified or alternative analytical procedures to accomplish this objective.

Methods Employed - In accordance with the above objective, a method of glutathione assay has been developed which is based on the spectrophotometric measurement of the rate of color formation at 412 mμ in a mixture comprising TPNH, yeast glutathione reductase, the sulfhydryl reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), and the sample to be analyzed. The formation of color at the stated wavelength is due to reduction of essentially colorless Ellman's reagent to an intensely colored product in the presence of a sulfhydryl-containing compound such as reduced glutathione. A suitable control mixture is employed to correct for the rate of reduction of Ellman's reagent in the absence of added glutathione.

Major Findings - The enzymatic procedure outlined above is based on the observation that glutathione can act catalytically in the reduction of Ellman's reagent by TPNH in the presence of glutathione reductase, i.e., a very small amount of glutathione can catalyze the reduction of a very large amount of reagent. The rate of this reduction depends in a strictly rectilinear manner on the amount of glutathione present in the mixture. Because of the catalytic role played by glutathione in this system the sensitivity of the procedure is 10 to 100 times that exhibited by previously published methods and is, as a consequence, suitable for the quantitative analysis of glutathione in tissue fluids (plasma, saliva, urine) normally containing very low levels (ca. 1 µg/ml) of the peptide. Furthermore, the method is unique in that it effectively measures total glutathione (i.e., GSH + GSSG) content. In addition, large excesses of other non-glutathione sulfhydryl compounds (e.g., cysteine) do not interfere.

Significance to Bio-medical Research and the Program of the Institute - The maintenance of adequate levels of reduced glutathione in plant and animal tissues is believed to be essential for normal cell function. At least one specific human abnormality, viz. primaquine sensitivity, is believed to originate from an impaired ability of the erythrocyte to maintain its structural integrity due to a lowered glutathione content. A convenient and reliable method for glutathione assay of erythrocytes might therefore be of some value as a diagnostic procedure in individuals with this genetic defect. The foregoing assay would be particularly suitable for this purpose inasmuch as its sensitivity and specificity permit the determination of blood glutathione levels essentially without preliminary treatment of the sample other than the preparation of a 1:100 hemolysate from as little as 10 µl of blood.

Proposed Course of Project - It is proposed to continue the characterization of the disulfide-reducing enzyme system of rat liver referred to under "Objectives".

Honors and Awards: None

Publications: None

Serial No. NIAMD-LBM-3
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzyme Induction

Previous Serial Number: NIAMD-LBM-9

Principal Investigator: Dr. Irwin G. Leder

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 2
Professional: 1
Others: 1

Project Description:

Objectives - To study the induction and control of enzyme systems in yeast and in bacteria.

Methods Employed - Induced permease systems are studied in various strains in E. coli. By means of radioactive substrates, the specificity and kinetics of pool formation and the properties of such pools are studied. In Salmonella typhimurium and in yeast, the repression and inhibition of the biosynthetic pathway for thiamine is studied with mutant strains of Salmonella and with a pure enzyme derived from yeast.

Major Findings - A. Permease accumulated pools of various substrates in E. coli have been found to be extremely sensitive to simple cold shock. Although cold shock does not impair either the viability of cells, nor their subsequent capacity to accumulate substrates, rapid temperature transitions from ambient to below 5° may result in the total loss of accumulated substrate within 15 seconds. The susceptibility of a pool to cold shock varies not only with the strain of E. coli and the osmotic composition of the medium, but with the nature of the carbohydrate used as carbon source during cell growth. It has been possible to demonstrate the reverse process; i.e., the rapid uptake of a labeled substrate under conditions of cold shock in the absence of a specific permease. These results suggest that the effect of cold shock may be to induce the formation of transient pores in the permeability barrier of the cell.

B. In yeast studies, the enzyme thiamine phosphate pyrophosphorylase has been purified to crystallinity from bakers' yeast. It migrates as a single component in electrophoresis with a molecular weight exceeding 200,000. The synthesis of this enzyme is repressed by thiamine, but only limited feedback inhibition by thiamine, thiamine phosphate or thiamine pyrophosphate has been observed. Procedures for the synthesis of crystalline substrates for this enzyme have been devised.

Significance to Bio-medical Research and the Program of the Institute - These studies will further our understanding of many aspects of mammalian cell biology such as hormonal action and viral infection which have as their locus control mechanisms and membrane structures of the cell. The structural and functional properties of the bacterial membrane may be similar not only to the mammalian cell membrane, but to that of such intracellular structures as the mitochondria and the nucleus.

Proposed Course of Project - The permease study will be extended to a cell free membrane system. In particular, the phosphorylation of TMG by two distinct and inducible permease systems will be studied both with respect to the action of permease specific competitive substrates and possible differences in phosphorylating agent. The purified thiamine synthetase from yeast will be studied to determine the nature of the subunit structure of this large molecule. The purified enzyme will also be used as a reagent in studying the synthesis of thiazole precursors in Salmonella mutants.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LBM-4
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemical Lesions in the Genetic Mucopolysaccharidoses

Previous Serial Number: NIAMD-LBM-11

Principal Investigator: Dr. Elizabeth F. Neufeld

Other Investigators: Dr. Joseph C. Fratantoni and Mrs. Clara W. Hall

Cooperating Units: None

Man Years:

Total: 3-1/2
Professional: 3
Other: 1/2

Project Description:

Objectives - To determine the biochemical defect(s) in the genetic disorders of mucopolysaccharide metabolism (Hurler, Hunter and Sanfilippo syndromes).

Methods Employed - Fibroblasts are cultured from skin biopsies of patients with the Hurler, Hunter or Sanfilippo syndromes, as well as from normal controls, for the study of sulfated mucopolysaccharide metabolism.

Major Findings - The kinetics of sulfated mucopolysaccharide turnover in normal fibroblasts show that about 2/3 of the newly-synthesized mucopolysaccharide (presumably, chondroitin sulfate B) is secreted into the medium while 1/3 is diverted to an intracellular pool where it is slowly degraded. Fibroblasts from Hurler and Hunter patients synthesize and secrete sulfated mucopolysaccharide at a normal rate; likewise, they fill the intracellular pool at a normal rate but fail to empty it because of inability to degrade the mucopolysaccharide.

Preliminary experiments have shown that mixing Hurler and Hunter fibroblasts causes a correction of the defect in both. The defect in Hurler fibroblasts is likewise corrected by the admixture of cells from Hunter, Sanfilippo and normal cultures, but not from another Hurler culture. These results, though incomplete, suggest that fibroblasts from each of the genetic forms lack a factor required to degrade mucopolysaccharides, which can be supplied (*via* the medium) by normal cells or by cells of the other two variants.

Significance to Bio-medical Research and the Program of the Institute - The genetic mucopolysaccharidoses, though rare in occurrence, cause severe mental retardation and early death. There is no effective treatment.

The findings described above imply the possibility of purifying factors which could replace those missing in the patients. The methods developed may also be useful for the classification of the disorders (often difficult by clinical criteria, because of overlap symptoms) and for the study of other metabolic diseases.

Proposed Course of Project - We shall attempt to identify precisely the steps at which mucopolysaccharide degradation is blocked, and to purify and identify the factors which can overcome the block.

Honors and Awards: None

Publications:

Fratantoni, J. C., Hall, C. W., and Neufeld, E. F.: The defect in Hurler's and Hunter's syndromes: faulty degradation of mucopolysaccharide. *Proc*. *Nat*. *Acad*. *Sci*. *U*. *S*. (in press).

Serial No. NIAMD-LBM-5
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Synthesis and Secretion of γ-Globulin

Previous Serial Number: NIAMD-LBM-7

Principal Investigator: Dr. Milton Kern

Other Investigators: Dr. Robert M. Swenson, Dr. Richard P. D'Amico
and Dr. Harvey J. Cohen

Cooperating Units: None

Man Years:

Total: 4-1/2
Professional: 4
Other: 1/2

Project Description:

Objectives - The purpose of this project is to study the nature of the intracellular mechanisms responsible for the secretion of γ-globulins by lymph node cells.

Methods Employed - (1) Lymph node cells were incubated with radioactive amino acids or monosaccharides to label γ-globulin in its protein or carbohydrate component, respectively. The cells were harvested, homogenized in 0.88 M sucrose and then separated into a cell sap and microsome fraction by differential centrifugation. γ-Globulin was solubilized from the microsome fraction by brief treatment with 0.01 N HCl - 0.88 M sucrose at 3^{o} followed by neutralization and centrifugation to remove microsome particles. The distribution of labeled γ-globulin between microsome and cell sap fractions was determined by specifically coprecipitating the γ-globulin with carrier rabbit γ-globulin and goat anti-rabbit-γ-globulin. In other experiments, cells incubated with radioactive substrates for varying lengths of time were separated from the extracellular fluid by centrifugation and lysed with deoxycholate. After removal of the particulate matter from the cell lysate by centrifugation at 100,000 x g, the intracellular and extracellular labeled γ-globulins were assayed by specific coprecipitation.

(2) The cell free incorporation of sugars into γ-globulin was studied by incubating microsomes derived from lymph node cells in the presence of appropriate sugar nucleotides. The carbohydrate labeled γ-globulin product was solubilized from the microsomes and assayed by the specific coprecipitation technique.

(3) Microsomes derived from lymph nodes of guinea pigs immunized with dinitrophenyl-protein possess firmly associated antibody specific for the DNP determinant. Such antibody can be dissociated by incubating microsomes in the presence of normal rabbit or guinea pig γ-globulin. The sites on antibody involved in binding to microsomes was studied by determining the effectiveness of chemically altered and proteolytically digested γ-globulin products for their ability to dissociate the microsome:antibody complex.

Major Findings - While radioactive amino acids are incorporated into intracellular γ-globulin without delay, the initial extracellular appearance of such γ-globulin requires about 20 minutes. Similar results were obtained when the carbohydrate component of γ-globulin was selectively labeled with H^3-galactose and H^3-glucosamine. Thus for γ-globulins labeled in their amino acid or carbohydrate component the curves for the initial appearance of intracellular and extracellular γ-globulin are parallel and separated by an interval of 20 minutes. These findings indicate that at least some of the galactose and glucosamine residues are acquired close to the time of synthesis of the polypeptide chains. In contrast it is clear that the single sialic acid residue of γ-globulin is acquired close to the moment of secretion rather than synthesis because the curves for the intracellular and extracellular appearance of sialic labeled γ-globulin are superimposable. When H^3-glucose was used to label the carbohydrate component, the labeled γ-globulin appeared extracellularly without delay. Since, in such experiments, the sialic acid was selectively removed prior to analysis, the absence of a secretory lag indicates that some sugars other than sialic acid are acquired relatively close to the time of secretion. These observations demonstrate that the carbohydrate component is slowly synthesized over the 20 minute period between the synthesis of the polypeptide chains of γ-globulin and the initial extracellular appearance of this protein.

The cell-free incorporation of C^{14}-galactose into a microsome associated, endogenous γ-globulin acceptor was demonstrated. Although the labeled γ-globulin sedimented with the microsomes, it could be solubilized from this structure by treatment with 0.01 N HCl, sonic oscillation or deoxycholate. The fact that greater than 80% of the solubilized, protein linked, radioactivity was accounted for as γ-globulin indicates that the product was compartmentalized within the microsomes. Sonic treatment of microsomes prior to the incorporation assay resulted in complete inhibition of the formation of C^{14}-galactose labeled γ-globulin without inhibition of incorporation into other glycoproteins. Thus it would appear that there is a special mechanism for the synthesis of the carbohydrate component of γ-globulin and that this mechanism is related to the fact that γ-globulin is the sole protein (glycoprotein) secreted by lymph node cells.

Anti-dinitrophenyl antibody associated with microsomes derived from appropriately immunized guinea pigs can be solubilized with normal γ-globulins and certain of its products obtained by chemical and enzymatic treatment. After digestion of normal γ-globulin with pepsin, an active peptide fraction was isolated having an estimated molecular wieght of 2,000-4,000. Periodate oxidation of normal γ-globulins, under conditions which destroy only sialic acid and fucose residues, resulted in a 5-fold enhancement of the ability of the treated γ-globulin to solubilize antibody from the microsomes. The reduction of a single disulfide bond of γ-globulin located at the center of the heavy chains yielded a product which was 1/5 as effective as the untreated protein with regard to its capacity to dissociate antibody. Direct reduction of the microsome:antibody complex also resulted in a loss in antibody. It should be noted that the reactive disulfide of γ-globulin is adjacent to a threonine residue bearing one of the carbohydrate chains. These results have been tentatively interpreted to suggest that microsomal antibody is a reflection of the processes involved in the acquisition of the carbohydrate component.

Significance to Bio-medical Research and the Program of the Institute - The abnormal γ-globulins and their subunits that have been observed in serum and urine may be a consequence of aberrations in the secretory mechanism, as well as the synthetic mechanism. Studies from this laboratory have elucidated several new parameters that define secretion of normal γ-globulin and provide a means for assessing the similarities and dissimilarities between a normal and abnormal cell. In addition, such parameters may also be useful in distinguishing whether the differentiation of lymphocytes to yield cells which produce and secrete γ-globulin develop the polypeptide synthesizing and secretory apparatus simultaneously or not.

Proposed Course of Project - The cell-free studies will be extended to determine the characteristics for acquisition of all known residues of the carbohydrate chain of γ-globulin in an attempt to relate this information to the results obtained with intact cells. Comparison studies will be made between mouse spleen and mouse myeloma cells with regard to the parameters known for the synthesis and secretion in order to assess the site of the lesion responsible for aberrant immunoglobulin production.

Honors and Awards: None

Publications:

Swenson, R. M., and Kern, M.: Synthesis and secretion of γ-globulin by lymph node cells. II. The intracellular segregation of amino acid-labeled and carbohydrate labeled γ-globulin. J. Biol. Chem. 242: 3242-3244, July 1967.

Swenson, R. M., and Kern, M.: Synthesis and secretion of γ-globulin by lymph node cells. III. The slow acquisition of the carbohydrate moiety of γ-globulin and its relationship to secretion. Proc. Nat. Acad. Sci. U. S. 59: 546-553, Feb. 1968.

Kern, M., and Swenson, R. M.: Biochemical studies on the intracellular events involved in the secretion of γ-globulin. Cold Spring Harbor Symp. on Quant. Biol. 32: 265-268, 1967.

D'Amico, R. P., and Kern, M.: Synthesis and secretion of γ-globulin by lymph node cells. IV. The cell-free incorporation of galactose into the carbohydrate component of γ-globulin. J. Biol. Chem. (in press).

Serial Number NIAMD-LBM-6
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Hormone-Dependent Differentiation of Mammary Gland *In Vitro*

Previous Serial Number: NIAMD-LBM-14

Principal Investigator: Dr. Yale J. Topper

Other Investigators: Dr. Joel J. Elias, Dr. Marie Green, Dr. Elinor S. Mills, Dr. Earl W. Sutherland III and Dr. Anthony E. Voytovich

Cooperating Units: None

Man Years:

Total: 7-1/2
Professional: 5-1/2
Others: 2

Project Description:

Objectives - To study the molecular and cytological phenomena involved in development of mammary gland.

Methods Employed - Mammary gland explants are cultured on synthetic media. Synthesis of specific milk proteins is followed by isolation of these materials after the explants have been pulsed with radioactive precursors. DNA and RNA synthesis are determined in the same way. The activities of various enzymes related to DNA synthesis are assayed by conventional methods. In some instances explants are treated with collagenase to remove fat cells; the residual cells are then studied using methods cited above. Cell proliferation is studied by means of mitotic indices and autoradiography. Cytology is examined with the light microscope and the electron microscope.

Major Findings - It has been established that the three hormones, insulin, hydrocortisone and prolactin, cannot elicit biochemical or morphological differentiation of parent mouse mammary epithelial cells. Rather, when epithelial cell division occurs in the presence of these hormones the daughter cells are differentiated. Partial resolution of this triple hormone system has been accomplished. Insulin is necessary and sufficient for the initiation of DNA synthesis in tissue derived from mature virgin and pregnant mice. Insulin is also required during the post-mitotic phase of the cell cycle. Hydrocortisone, in this system, must be present during the proliferative phases of the cell cycle. This is at least one of the reasons why the overall process is dependent on cell division. Prolactin can act post-mitotically. Thus two types of mammary epithelial cells can be formed _in vitro_, neither of which is capable of synthesizing milk proteins. Cells formed in the presence of insulin alone cannot subsequently respond, in terms of milk-protein synthesis, to the addition of hydrocortisone and prolactin. Cells formed in the presence of insulin and hydrocortisone can respond to the post-mitotic addition of prolactin. The induction of casein synthesis by the post-mitotic action of prolactin appears to start from what approaches a zero-baseline synthetic capacity.

The initiation of epithelial cell DNA synthesis by insulin is due, at least in part, to the appearance of DNA polymerase activity. Androgens inhibit the overall differentiation process by inhibiting DNA synthesis by mammary epithelial cells.

Explants from the mammary glands of 3-week-old mice can be induced to synthesize casein _in vitro_ in the absence of lobuloalveolar development. Maximum biochemical differentiation requires the presence of insulin, hydrocortisone, and prolactin in the culture medium. In contrast to explants from adult mice, the mammary epithelium of immature animals undergoes DNA synthesis and mitosis _in vitro_ in the absence of exogenous insulin; however, such proliferation does not lead to the formation of differentiated daughter cells. Insulin acts in at least two ways during the proliferative phases of the cell cycle of differentiating mammary epithelial cells.

It was previously reported that both insulin and prolactin are required during the post-mitotic phases of the process. It now appears that, during this period, prolactin acts before insulin.

Significance to Bio-medical Research and the Program of the Institute - Studies on the mechanisms involved in normal tissue development are necessary for eventual understanding of various disease states.

Proposed Course of Project - Emphasis will be placed on determination of the actions of the individual hormones involved in this system. The role of cell division will be studied further.

Honors and Awards: None

Publications:

Lockwood, D. H., Stockdale, F. E., and Topper, Y. J.: Hormone-dependent differentiation of mammary gland: The sequence of action of hormones in relation to the cell cycle. _Science_ 156: 945-946, May 1967.

Turkington, R. W., Juergens, W. G., and Topper, Y. J.: Steroid structural requirements for mammary gland differentiation *in vitro*. *Endocrinol*. 80: 1139-1142, June 1967.

Lockwood, D. H., Voytovich, A. E., Stockdale, F. E., and Topper, Y. J.: Insulin-dependent DNA synthesis in mammary epithelial cells *in vitro*. *Proc*. *Nat*. *Acad*. *Sci*. *U*. *S*. 58: 658-664, Aug. 1967.

Turkington, R. W., Lockwood, D. H., and Topper, Y. J.: The induction of milk protein synthesis in post-mitotic mammary epithelial cells exposed to prolactin. *Biochim*. *Biophys*. *Acta* 148: 475-480, Nov. 1967.

Voytovich, A. E., and Topper, Y. J.: Hormone dependent differentiation of immature mouse mammary gland *in vitro*. *Science* 158: 1326-1327, Dec. 1967.

Topper, Y. J.: Multiple hormone interactions related to the growth and differentiation of mammary gland *in vitro*. *Trans*. *N*. *Y*. *Acad*. *Sci*. (in press).

Serial No. NIAMD-LBM-7
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Carbohydrate Metabolism

Previous Serial Number: NIAMD-LBM-1

Principal Investigator: Dr. Gilbert Ashwell

Other Investigators: Dr. Jean Hickman
Dr. Lee Van Lenten
Dr. Kenneth Klatt

Cooperating Units: Dr. Anatol Morell, Department of Medicine, Albert Einstein College of Medicine, New York, N. Y.

Man Years

Total: 5
Professional: 4
Other: 1

Project Description:

Objectives:

The major objective of this project has been a study of the role of the carbohydrate moiety of glycoproteins in determining the structure, function and metabolism of these macromolecules. Additional information is sought concerning the unique mechanisms of biosynthesis.

Methods Employed:

In general, the methods employed involve the use of highly purified enzymes to effect alteration or removal of specific monosaccharides on the protein. In some cases, radioactive labelling has been utilized to trace the fate of the modified molecule. Both *in vivo* and *in vitro* analyses are now carried out.

Major Findings:

Continuation of the studies on ceruloplasmin (with Professor Morell at Albert Einstein College of Medicine) have resulted in several findings of basic interest and import for the understanding of the pathology of Wilson's disease.

Major Findings (continued):

Removal of one or more of the sialic acid residues on the various carbohydrate chains attached to the protein results in the exposure of a new, nonreducing sugar, D-galactose. In the presence of this terminal monosaccharide, the circulating protein is rapidly and quantitatively removed from the blood and accumulated within the parenchymal cells of the liver. The rapidity of this translocation appears to be uniquely dependent upon the presence of intact, terminal galactosyl residues; alteration or removal of this sugar obliterates the phenomenon.

Subsequent studies, directed towards evaluation of the role of the enzyme, sialyl transferase, in the etiology of the disease state, revealed a markedly lower enzyme titer in biopsy samples of livers from patients suffering from Wilson's disease. This finding, in turn, has been traced to the presence of an inhibitor which has been tentatively identified as the unsaturated fatty acid, oleic acid. [Dr. Jean Hickman]

An alternate approach to a study of the role of protein-bound carbohydrates is being explored with the use of ribonuclease A and B as model compounds. The latter enzyme, B, is identical to A in all known parameters (activity, molecular weight, amino acid composition, etc.) with the exception that RNase B contains a carbohydrate chain consisting of 6 residues of D-mannose and 2 residues of N-acetyl-D-glucosamine.

Sequential enzymatic removal of these monosaccharide units, followed by extensive purification of the resulting protein species, has led to the isolation of a protein tentatively identified as RNase A. Efforts are currently underway to isolate and purify the linkage enzyme responsible for cleavage of the primary bond between N-acetylglucosamine and asparagine. [Dr. Lee Van Lenten]

Proposed Course of Project:

With the demonstration that the livers from patients with Wilson's disease exhibit an unusually high concentration of the fatty acids--together with the finding that the synthesis of ceruloplasmin is markedly affected by oleic acid, a possible defect in the fatty acid metabolism of these patients will be sought. Identification of such a metabolic block would provide new insight into the nature of the basic lesion in this genetic disease.

Publications:

Morell, A. G., Irvine, R. A., Sternlieb, I., Scheinberg, I. H., and Ashwell, G.: Physical and chemical studies on ceruloplasmin V. Metabolic studies on sialic acid-free ceruloplasmin *in vivo*. *J. Biol. Chem.* 243: 155-159, Jan. 1968.

Serial No. NIAMD-LBM-8
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biosynthesis of Storage and Structural Polysaccharides and its Regulation

Previous Serial Number: None

Principal Investigator: Dr. Enrico Cabib

Other Investigators: Dr. Lucia B. Rothman

Cooperating Units: None

Man Years

Total:	2-1/4
Professional:	1/2
Other:	3/4

Project Description:

Objectives:

A primary aim of this project is to ascertain the mechanisms by which polysaccharides are enzymatically synthesized, and assembled into the structure of a cell wall. Another line of investigation is directed to elucidate the systems by which the formation of complex sugars is regulated.

Methods Employed:

Most methods used were conventional enzymatic procedures. The enzymes involved in the biosynthesis of polysaccharides were studied in a particulate preparation, obtained by lysing yeast protoplasts. The changes in concentration of several metabolites in yeast under different conditions were determined by spectrophotometric methods, with the help of auxiliary enzymes.

Major Findings:

1. The cell wall of yeast contains two major carbohydrate components, mannan and glucan, and a minor component, chitin. Recent investigations have located the chitin in the bud scar region of the cell wall. These reports have found at least partial confirmation in our laboratory,

Major Findings (continued):

through the isolation of an hexosamine-rich fraction from the cell wall of the yeast strain used in our investigations. It has now been found that a particulate preparation from yeast protoplasts catalyzes the incorporation of C^{14}-acetylglucosamine from UDP-acetylglucosamine into a material insoluble in 66% ethanol. The radioactive material has been provisionally identified as chitin by its insolubility in acid and alkali as well as by the release of acetylglucosamine after treatment with a chitinase and of glucosamine after acid hydrolysis. The enzymatic activity has an absolute requirement for Mg^{++} or Mn^{++} and is also activated about five-fold by 0.04 M free acetylglucosamine. Glucose and glycerol at very high concentrations, but not acetylgalactosamine, can substitute for acetylglucosamine.

Protoplast lysate supernatants or whole cell extracts inhibit the activity of the chitin synthetase. At least part of the inhibitory power seems to reside in heat-stable, nondialyzable material. The inhibition is eliminated by treatment of the (se) substance(s) with alkali or with proteases, but not with ribonuclease. Thus, the inhibitor appears to be of proteinaceous nature and is being further investigated.

2. In a previous study it was shown that yeast and muscle glucogen synthetases are allosterically inhibited by ATP and ADP and that this inhibition is reversed by glucose-6-phosphate. In order to investigate the significance of these findings for the physiological regulation of glycogen synthesis, studies have been undertaken to correlate the change in concentration of the relevant metabolites "in vivo" with the rate of formation of the polysaccharide. Most of the work carried out in the period covered by the present report has been concerned with choosing, trying and modifying methods for the extraction and assay of glycogen, glucose, glucose-6-P, ATP, ADP and AMP from yeast. Preliminary results show that a rapid formation of glycogen is correlated with a high concentration of glucose-6-P, with little variation in the sum of ATP + ADP. The inhibition of glycogen synthesis "in vivo" produced by ammonium ions is paralleled by a sharp decrease in glucose-6-P concentration.

Significance to NIAMD Research:

The formation of a cell wall poses fundamental problems, such as transfer of material across cell membranes, assembly of a biological structure and mechanisms of cellular growth and reproduction. In addition, the cell wall is a structure that is present in many pathogenic agents but not in their hosts; accordingly, the process of its formation has been found to be the target of several antibiotics which are relatively harmless for the infected organism.

The regulation of glycogen biosynthesis is of primary importance for the distribution of energy in many cells. Our recent findings that the

Significance to NIAMD Research (continued):

yeast and muscle glycogen synthetases resemble each other in several aspects of their regulation testifies to the generality of the mechanisms being investigated.

Proposed Course of Project:

Previous and present work has established the pathways of formation of two of the three polysaccharides of the yeast cell wall, i.e., mannan and chitin, while the mechanism of synthesis of glycan is still unknown. It is proposed to continue the study of the enzymatic systems concerned with the synthesis of these polysaccharides, in the hope of obtaining a better insight in the process of wall formation and of budding. It is also our intention to continue the investigation on the regulation of glycogen biosynthesis and, if possible, to extend these studies to the formation of trehalose, the other major storage carbohydrate of yeast.

Serial No. NIAMD-LBM-9
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzymatic Utilization of Model Compounds

Previous Serial Number: NIAMD-LBM-3

Principal Investigator: Dr. William B. Jakoby

Other Investigators: Dr. William Scher
Dr. Robert H. Allen
Dr. Julia F. Clark

Cooperating Units: Dr. Leonard D. Kohn

Man Years

Total:	5
Professional:	4
Other:	1

Project Description:

Objectives:

These investigations are concerned with the reactivity of a variety of chemical groupings in enzyme-catalyzed reactions. Choices are such as to lead to investigations of the general phenomena of enzyme mechanisms.

Methods Employed:

The usual enzymological techniques are used. By use of the enrichment culture technique, microorganisms may be selected with the ability to produce relatively large amounts of enzyme specific for a given substrate.

Major Findings:

These studies have continued a two-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups and, as well, on those features of proteins responsible for specific catalysis. In the former category are the elucidation of pathways for the bacterial synthesis and degradation of tartaric acid (epoxysuccinate → tartrate → oxaloglycolate → glycerate + CO_2) [Dr. R. H. Allen and Dr. L. D. Kohn] and for maleic acid (maleate → fumarate → malate) [Dr. W. Scher].

Major Findings (continued):

Contributions in the latter group involve preparative and analytical methodology as well as an analysis of structural and catalytic properties of aldehyde dehydrogenase. [Dr. J. F. Clark].

The last of these projects, the properties of a yeast aldehyde dehydrogenase, hazard is being studied in detail. During isolation, the protein is extremely sensitive to proteolysis by enzymes present in the extracts and it has only been recently that preparations with a single amino acid end group have been obtained. Thus, although a crystalline and apparently homogeneous preparation was obtained two years ago it is only now that what may be termed microheterogeneity has been eliminated. The active enzyme is a relatively large protein composed of an as yet undetermined number of monomeric units. The dissociation and reassociation of monomers is presently being analyzed in terms of kinetics of both changes in size and catalytic activity.

Significance to NIAMD Research:

Studies of enzyme mechanism and of patterns of metabolism relate directly to the fundamentals of all biological activity.

Proposed Course of Project:

It is expected that investigations dealing with the above-noted projects will be continued and a study of other model reactions will be initiated.

Publications:

Steinman, C. R., and Jakoby, W. B.: Yeast aldehyde dehydrogenase II. Properties of the homogeneous enzyme preparation. J. Biol. Chem. 243: 730-734, Feb. 1968.

Kohn, L. D., and Jakoby, W. B.: Tartaric acid metabolism III. The formation of glyceric acid. J. Biol. Chem. 243: 2465-2471, May 1968.

Kohn, L. D., and Jakoby, W. B.: Tartaric acid metabolism IV. Crystalline L-malic dehydrogenase from Pseudomonas acidovorans. J. Biol. Chem. 243: 2472-2478, May 1968.

Kohn, L. D., Packman, P. M., Allen, R. H., and Jakoby, W. B.: Tartaric acid metabolism V. Crystalline tartrate dehydrogenase. J. Biol. Chem. 243: 2479-2485, May 1968.

Kohn, L. D., and Jakoby, W. B.: Tartaric acid metabolism VI. Crystalline oxaloglycalate reductive decarboxylase. J. Biol. Chem. 243: 2486-2493, May 1968.

Jakoby, W. B.: A technique for the crystallization of proteins. Anal. Biochem., in press

Packman, L. D., and Jakoby, W. B.: Crystalline quinolinate phosphoribosyltransferase. In Tabor, H., and Tabor, C. W. (Eds.): Methods in Enzymology, vol 14, in press.

Scher, W., and Jakoby, W. B.: Maleic acid. In Bergmeyer, H. U. (Ed.): Methods of Enzymatic Analysis, 2 ed., in press.

Serial No. NIAMD-LBM-10
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis, and Biological Significance

Previous Serial Number: NIAMD-LBM-4

Principal Investigator: Dr. Marie N. Lipsett

Other Investigators: Dr. Elaine Kaufman
Dr. Jack Abrell

Cooperating Units: None

Man Years

Total: 3-1/2
Professional: 2-1/2
Other: 1

Project Description:

Objectives:

The objective of this project is the study of the sulfur-containing nucleotides found in the tRNA of some species. This is pursued in three main directions: 1) The biosynthetic pathways involved in the formation of these thionucleotides; 2) the chemistry of 4-thiouridylate and its disulfide, and 3) the study of such disulfides in the tRNA molecule and the role of sulfur in the structure and function of tRNA.

Methods Employed:

Conventional procedures have been used for the salt- and column-fractionations of the sulfurtransferase. Nucleotide separations are carried out on alkaline digests using DEAE chromatography, electrophoresis, etc. The secondary structure of tRNA is investigated with the use of absorption spectra, heat and urea denaturation, sedimentation behavior, etc.

Major Findings:

1. The purification of the sulfurtransferase which transforms tRNA uridine into 4-thiouridine was continued. The previous purification

Major Findings (continued):

method proved somewhat unreliable, and a second scheme utilizing a dextran partition step was evolved. With this procedure, our initial purification is approximately 50 times and a further step gives promise of raising the over all purification to about 2000 times. This number is somewhat misleading because of a simultaneous activation.

2. Further work on the state of the 4-thiouridine (4-TU) residues in tRNA, using urea or guanidinium chloride solutions as solvents during thermal denaturation, indicates that about one-third of the 4-TU in unfractionated tRNA is stacked rather than hydrogen-bonded in the structure. Since we have found that almost all of the 4-TU occurs next to purine bases, this may represent a purine-4-TU stacking interaction.

Significance to NIAMD Research:

The tertiary structure of the tRNA molecule has been found to influence its biological activity profoundly. 4-Thiouridine, by virtue of its unique element and spectrum, offers a convenient tool for exploring the structural conformations of tRNA and their relationship to regulatory mechanisms for protein synthesis.

Proposed Course of Project:

The purification of the sulfurtransferase will be carried on until the enzyme is sufficiently pure to permit mechanism studies. It is hoped that knowledge about the specificities of the enzyme will throw light both on the tRNA base sequences required to permit thiolation of specific uridines and on the secondary structure of tRNA itself.

Purified tyrosine tRNA will be isolated from E. coli. Since this tRNA species contains two 4-TU per molecule, it will be useful for studying the relationship between biological activity and secondary structure in tRNA, as modified by disulfide formation, alkylation, desulfuration, etc. In addition, the base sequence in the neighborhood of the 4-TU residues will be determined, to give an indication of the acceptor sequence for the sulfurtransferase. This problem is also being attacked by production of sulfur-poor tRNA, using a cystine-requiring mutant and chloramphenicol.

Publications:

Lipsett, M. N.: Disulfide bonds in sRNA. Cold Spring Harbor Symp. on Quant. Biol. and Med., 31: 449-455, 1966.

Lipsett, M. N.: The isolation of 4-thiouridylate disulfide from oxidized transfer ribonucleic acid of Escherichia coli. J. Biol. Chem. 242: 4067-4071, Sept. 1967.

Publications (continued):

Lipsett, M. N., and Doctor, B. P.: Studies on tyrosine transfer ribo--nucleic acid, a sulfur-rich species from *Escherichia coli*. *J. Biol. Chem*. 424: 4072-4077, Sept. 1967.

Serial No. NIAMD-LBM-11
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemical and Enzymic Studies Related to the Structure, Metabolism and Function of Ribonucleic Acid

Previous Serial Number: NIAMD-LBM-5

Principal Investigator: Dr. Maxine F. Singer

Other Investigators: Dr. David M. Logan, Postdoctoral Fellow, Jane Coffin Childs Memorial Fund for Medical Research
Dr. James Castles, Research Associate, NIAMD
Dr. Claude Klee
Dr. Robb Moses
Mr. George Tolbert, NIAMD, Summer Employee

Cooperating Units: None

Man Years

Total:	5-1/2
Professional:	4-1/4
Other:	1-1/4

Project Description:

Objectives:

1. To extend earlier studies on the processive exonucleolytic degradation of polyribonucleotides, in particular with the exonuclease of Lactobacillus arabinosus.

2. To investigate the mechanisms by which the activities of bacterial RNase II and polynucleotide phosphorylase are controlled within cells, and to gain further insight into the physiological role of these enzymes.

3. To study the chemistry of polynucleotide phosphorylase purified from Micrococcus luteus (formerly Micrococcus lysodeikticus.) In particular, to try to understand how the various structural forms of the enzyme are related to functional modifications.

Methods Employed:

To a large degree both our preparative and analytical work depends on the use of enzymes as specific reagents. The purely chemical techniques are of limited value with nucleic acids. Instead, a large inventory of highly specific enzymes is maintained. The enzymes are either prepared in the lab or in some cases, obtained commercially.

During the past year we have increasingly relied on gel filtration for the purification of both proteins and nucleic acids.

In the studies on polynucleotide phosphorylase disk gel electrophoresis has proven extremely valuable. In large measure this results from the use of excellent procedure for detecting the polymerizing activity of the enzyme on the intact gels.

In addition, standard biochemical, radiochemical and physical methods were used.

Major Findings:

1. The exoribonuclease purified previously from Lactobacillus arabinosus was shown to be quite similar, in enzymatic properties, to E. coli RNase II. Thus, it is stimulated by K^+ or NH_4^+ ions, it requires Mg^{++}, it is specific for single-stranded polyribonucleotides, it produces 5'-nucleoside monophosphates and it degrades polymers in a processive fashion, that is, it tends to degrade a single chain to completion prior to initiating hydrolysis of another chain. The experiments used to prove the latter point were identical to those we used previously with the E. coli RNase II.

2. Both E. coli RNase II and polynucleotide phosphorylase have been implicated as enzymes responsible for the breakdown of messenger RNA in vivo and in vitro. The question then arises as to what protects messenger RNA from degradation. We have been able to show that mRNA is partially protected when it is involved in a stable complex with ribosomes. At 37°, using poly U (synthetic messenger RNA) in a complex with ribosomes is readily degraded by RNase II. Stabilization of the complex by any of the following conditions leads to some protection: 1) decreased temperature, i.e., 10°, 2) addition of phenylalanine-transfer RNA to the complex, 3) fixation of the poly U to the ribosomes by treatment with formaldehyde (the poly U itself is thereby not modified but it is trapped onto the ribosome even at 10^{-4} M Mg^{++}). Our current interpretation of these results is based on the extent of protection in each case and on the known properties of RNase II. The enzyme begins degrading at the free terminal C-3'-hydroxyl end of the poly U and continues until it meets a point of stable ribosome binding, when it stops. The remainder of the poly U is protected. Similar experimental results with polynucleotide phosphorylase suggest a similar interpretation.

Major Findings (continued):

A mutant of E. coli (113 B) which lacks RNase I, is presumed to have a modified polynucleotide phosphorylase, and a temperature-sensitive RNase II was made available to us by Dr. Walter Gilbert of Harvard. In agreement with the work of others, we found that the phosphorylase has the following unusual properties: 1) it is more active with Mn^{++}; 2) it occurs in 113 B in at least two forms of differing molecular weight; 3) although polymerization of ADP is catalyzed readily, the enzyme barely catalyzes the back reaction (the phosphorolysis of poly A). We found that the RNase II of 113 B is indeed more sensitive to elevated temperature than the RNase II of normal E. coli. The temperature at which 50% inactivation occurs ofter 5 minutes' heating is 6 to 7° lower for the mutant enzyme. This is not caused by a protector in normal extracts or an inactivating factor in the mutant extracts. In other properties the two mutant enzymes are identical.

3. In last year's report, under Major Findings (paragraph 2), we presented an interpretation of preliminary data obtained with pure polynucleotide phosphorylase. The preliminary data have proven reproducible and have been extended. On the other hand, the preliminary interpretation has proven to be erroneous. The new data are presented here along with some new and more promising interpretations.

As isolated from Micrococcus luteus (formerly M. lysodeikticus) polynucleotide phosphorylase is homogeneous with respect to molecular weight (255,000 s_{20w}, 92). In guanidine-HCl, in the presence of -SH, it breaks into subunits of molecular weight 64,000. On disk gel electrophoresis the pure enzyme shows at least six protein bands each of which coincides with a band of activity. These forms can be partly resolved by chromatography on hydroxylapatite and DEAE. The enzyme catalyzes polymerization with no requirement for oligonucleotide primer. It can be oxidized, under mild conditions, to give form that does require primer.

Another, and distinct primer-dependent form is obtainable by limited tryptic hydrolysis. This enzyme has a molecular weight of 220,000 (58,000 in guanidine-HCl) and an s_{20w} of 9.0. Concomitant with tryptic hydrolysis all of the original electrophoretic bands are converted to a new, single band containing all the protein and activity. This form becomes primer independent again upon reduction with sulfhydryl compounds. Reduced enzyme can be reconverted to primer dependence by the common sulfhydryl inhibitors. These manipulations do not significantly affect the overall ability of the enzyme to catalyze polymer synthesis (in the presence of primer) or phosphorolytic degradation of polyribonucleotides.

The ability to initiate polymerization appears to be related to the nature of one or more sulfur residues on the protein. It is possible that during trypsin treatment these residues become easily oxidizable, yielding a primer-dependent form. The loss of a part of the polypeptide would then be only indirectly responsible for the functional modification. It is possible that the functional interconversions are indeed most directly influenced by conformational changes accompanying the chemical modifications.

Significance to NIAMD Research:

Both polynucleotide phosphorylase and *E. coli* RNase II may be involved in the degradation of messenger RNA. A detailed understanding of the properties and functions of these enzymes is essential to an understanding of the cellular control of protein synthesis.

The central role of nucleic acids in cellular function is a fact. Enzymes that synthesize these polymers are therefore of basic importance. In spite of a decade of brilliant work in many laboratories, the details of the reactions catalyzed by nucleic acid polymerases are still obscure. Polynucleotide phosphorylase is one of these enzymes. Understanding its mechanism of action is then of great interest.

Proposed Course of Project:

The work on polynucleotide phosphorylase will be extended. Particular effort will be applied to obtain large amounts of pure enzyme. The work up to now has been limited by the lack of sufficient enzyme. Study of the protein structure of the various forms of the enzyme will then be possible. Conformational studies will also be initiated.

We are interested in a more detailed comparison of the normal and mutant RNase II. In addition, the chemistry of the processive mechanism of degradation will be studied.

Publications:

Klee, C. B.: Structural alterations of polynucleotide phosphorylase leading to primer dependence. *J. Biol. Chem.* 242: 3579-3580, August 1967.

Logan, D. M., and Singer, M. F.: New procedures for the preparation of complexes of ribosomes with polyuridylic acid. *Biochemistry* 6: 2678-2685, October 1967.

Klee, C. B., and Singer, M. F.: A convenient method for the preparation of primer-dependent polynucleotide phosphorylase from *Micrococcus lysodeikticus*. *Biochem. Biophys. Res. Commun.* 29: 356-361, Nov. 1967.

Nossal, N. G., and Singer, M. F.: The processive degradation of individual polyribonucleotide chains I. *E. coli* ribonuclease II. *J. Biol. Chem.* 243: 913-922, March 1968.

Publications (continued):

Klee, C. B., and Singer, M.F.: The processive degradation of individual polyribonucleotide chains II. *Micrococcus lysodeikticus* polynucleotide *J. Biol. Chem*. 243: 923-927, March 1968.

Singer, M. F.: *In vitro* synthesis of DNA: A perspective on Research. 2. The scientific aspects, *Science* 158: 1550, 1967.

Singer, M. F.: The MIT symposium on american women in science and engineering. In Mattfeld, J. A., and Van Aken, C. G. (Eds): *Bulletin of the Atomic Scientists*. Cambridge, 1967, pp. 57-58.

Serial No. NIAMD-LBM-12
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biochemical Studies of Lysogeny in _E. coli_ K12 and Enzyme Changes in Vaccinia infected Hela Cells

Previous Serial Number: NIAMD-LBM-6

Principal Investigator: Dr. Arthur Weissbach

Other Investigators: Mr. William E. Pricer, Jr.
Dr. Lois Salzman
Dr. Robert Shuster
Dr. Kenneth Berns

Cooperating Units: Dr. Paul Bartl, Department of Biophysics, Johns Hopkins University, Baltimore, Maryland
Dr. Norman Salzman, NIAMD-NIH, Bethesda, Maryland

Man Years

Total:	5-1/2
Professional:	4-1/2
Other:	1

Project Description:

Objectives:

1. To study the enzymatic and physical steps which control the establishment of lysogeny in bacteria and the induction of temperate phage formation.

2. To outline the enzymatic steps involved in the formation of mature bacteriophage and correlate this information with the known, genetically mapped, defective lysogenic bacteriophage mutants.

3. To study the intermediates involved in the biosynthesis of λ phage DNA and their role in the development of intact λ phage.

4. To study a new DNA polymerase synthesized by Vaccinia virus infection.

Methods Employed:

1. The preparation, isolation, and characterization of circular and intermediate concatenated DNA molecules is in progress.

2. Procedures for the growth and/or isolation of large amounts of *E. coli*, bacteriophage λ, hela cells, Vaccinia virus enzyme extracts, and various DNA's are being used.

3. Chemical and physical methods for the induction of lysogenic bacteria are used.

4. Various standard enzymological, microbiological, and isotopic techniques are employed.

Work in progress involves the following:

1. A study of the synthesis, structure, and biochemical significance of the concatenated, circular, and linear forms of λ phage DNA which are intermediates in the formation of mature virus. These DNA species are being isolated and their structure and role in the replication of phage DNA are being investigated.

2. The formation of virus-specific DNA polymerase in hela cells infected with Vaccinia virus.

3. The ability of various defective lysogenic K12λ strains to synthesize enzymes necessary for the synthesis and degradation of λ DNA replicative forms is being studied.

Major Findings:

1. Electron microscope studies with Dr. Paul Bartl have shown that the replicative forms of λ DNA include concatenated molecules 2 - 6 times the size of the mature λ DNA.

Circular λ DNA molecules, both supercoiled and open forms, are also important replicative intermediates. Circular structures 2 - 3 times the size of monomeric λ circles have been found and may be of significance in replication and/or recombination. [Drs. Salzman and Bartl]

2. A new DNase capable of attacking and destroying the twisted supercoil form of circular λ DNA has been found in λ infected cells. This enzyme is a phage directed enzyme and has only been detected in thymine-requiring cells which are unable to synthesize DNA under thymineless conditions. The properties and genetic control of this enzyme are being studied. [Dr. Robert Shuster]

Major Findings (continued):

3. DNA polymerase from hela cells has been purified several-hundred fold and its properties studied. The most significant finding is that this polymerase prefers single stranded DNA as a template and is unable to use Vaccinia DNA as a template. Attempts are in progress to isolate a new DNA polymerase from Vaccinia infected hela cells and study its properties. [Dr. K. Berns]

Significance to NIAMD Research:

Any clues to the control and regulatory mechanisms governing bacterial-viral relationships would be of general importance in understanding control mechanisms in all cellular activities.

Proposed Course of Project:

To depart.

Publications:

Salzman, L. A., and Weissbach, A.: Formation of intermediates in the replication of phage λ DNA. J. Mol. Biol. 28: 53-70, Nov. 1967.

Shuster, R. C., Breitman, T. R., and Weissbach, A.: The failure of three temperate coliphages to direct the synthesis of new λ type deoxyribonuclease. J. Biol. Chem. 242: 3723-3725, Aug. 1967.

Weissbach, A., and Salzman, L. A.: The biosynthesis of phage λ DNA: The structure of the first intermediate. Proc. Nat. Acad. Sci. 58: 1096-1101, Sept. 1967.

Salzman, L. A., and Weissbach, A.: Deoxyribonucleic acid replication in λ bacteriophage mutants. J. of Virology 2: 118-123, Feb. 1968.

ANNUAL REPORT SUMMARY

LABORATORY OF CHEMISTRY

National Institute of Arthritis and Metabolic Diseases

SECTION ON BIOCHEMICAL MECHANISMS

Oxidation Mechanisms in Mitochondrial Systems

In continuation of studies on chemical models for oxidative phosphorylation and conservation of oxidative energy, it has now been shown that the oxidation of a hydroquinone monoacetate, in the presence of halide ion in inert solvents, leads to the formation of the corresponding acyl halide. Thus, it appears possible to trap and conserve energy at the highest free energy level known for stable organic compounds. The most speculative aspect of a scheme previously proposed for oxidative phosphorylation, at the Coenzyme Q or Vitamin K step, requires the spontaneous esterification of a phenolic hydroxyl with a protein carboxyl group. Literature data indicate that the phenyl acetate content of a mixture of phenol and acetic acid, at equilibrium, would be very small. When the same reaction is studied in an intramolecular case, esterification proceeds to completion at pH 4-5, with a half reaction time of 15 minutes. Thus, the postulate of an esterification between reduced Coenzyme Q and an enzyme carboxyl is not unreasonable. Current research is devoted toward demonstration of the proposed scheme in mitochondrial systems and toward the development of alternate pathways for energy conservation (L. A. Cohen and J. W. Thanassi).

Selective Reduction of N-Terminal Pyroglutamyl Peptides

A variety of natural peptides and proteins have been discovered which have a pyrrolidone (pyroglutamyl) residue at the N-terminal. The non-basicity of such residues precludes the use of standard techniques for N-terminal identification or for amino acid sequencing. Reduction of the peptide with diborane in tetramethylurea solution (0°) results in partial conversion of the pyrrolidone ring to proline. It then becomes possible, by demonstration of a new N-terminal proline, to identify the original N-terminal residue and to initiate sequence studies. Although the reduction is neither quantitative nor absolutely specific, qualitative identification may be performed quite simply and rapidly. To date, the method has been tested on gastrin and several synthetic peptides (L. A. Cohen and S. Takahashi).

Fluorophosphates as Enzyme Substrates

In contrast to the highly reactive and toxic fluorophosphate diesters (such as diisopropyl fluorophosphate), fluorophosphate monoesters are stable and non-toxic. By use of a unique reaction between phosphates, fluorodinitrobenzene and triethylamine, several fluorophosphate analogues of natural triose and hexosephosphate substrates have been synthesized, with the intention of using them for irreversible infinity labeling of aldolase and various phosphatases. Surprisingly, such analogues not only fail to inhibit their respective enzymes but appear to be accepted and utilized in the normal fashion. Considerable significance may be attached to the possibility of getting fluorophosphate incorporated into DNA and RNA by the normal enzymatic pathways; studies along these lines are in progress (L. A. Cohen and S. Milstien).

Halonitroimidazoles as Selective Protein Reagents

Activated haloimidazoles, such as 4-bromo-5-nitroimidazole, react specifically with sulfhydryl groups of proteins, sulfur replacing the halogen atom. The resulting colored derivatives are useful in three ways: (1) enzymatic degradation of a blocked protein leads to peptide fragments; the location of cysteine residues in the original sequence can be ascertained by visual observation of color in the fragments; (2) since the absorption spectrum of the nitroimidazole system is fairly sensitive to the polarity in the vicinity of the cysteine residue; (3) since the pK of the imidazole NH is 5-6, the heterocyclic system is also sensitive to the ionic character of the environment; thus, conformational and electrostatic changes resulting from combination of enzyme and usbstrate should be spectroscopically detectable as changes in the degree of ionization of the nitroimidazole. The various uses of the "reporter" chromophore are being investigated with several enzymes (L. A. Cohen and W. Schreiber).

Reagents for Sequential Degradation of Proteins and Models for Proteolytic Enzymes

In further exploration of fluoronitrobenzimidazoles as peptide reagents, the peptide bond of 1,2-dimethyl-5,7-dinitrobenzimidazolyl-4-alanylglycine has been found to hydrolyze, at 50°, 200,000 times as fast as that of 2,4-dinitrophenylalanylglycine. Even more impressive results have been obtained with 2-amino-4,6-dinitrophenyl peptides: the rate of peptide cleavage, at 25° and pH 7-8, is too fast to measure by normal techniques and probably exceeds the rate of enzyme proteolysis. The latter constitutes the most efficient model of enzyme proteolysis discovered to date. Studies of the mechanism by which the intramolecular catalysis occurs suggest that enzymatic hydrolysis of esters and amides do not necessarily occur by parallel mechanisms and that the two types of substrates may involve entirely different catalytic sites on the enzyme. It is especially noteworthy that bases with pK values as low as 2 or 3 are extremely powerful catalysts in their protonated forms.

The same reagents have been explored for end-group identification and amino acid sequence determination. Although certain obstacles remain to be overcome, the rapidity and technical ease with which degradation may be accomplished make these reagents competitive with those currently in use for analytical purposes (L. A. Cohen and K. L. Kirk).

Measurement of Enzyme Kinetics by Fluoride Ion Assay

A fluoride-ion sensitive electrode has been applied to enzyme systems. Whenever a substrate can be utilized which releases fluoride ion as a result of reaction with an enzyme, the course of the reaction and enzyme activity can be determined by inserting the highly sensitive fluoride electrode in the reaction vessel. Ions such as phosphate and sulfate, which interfere with standard fluoride analyses, do not affect the electrode system (L. A. Cohen and S. Milstien).

SECTION ON CARBOHYDRATES

Steric Control in the Synthesis of Nucleosides

A careful examination of the use of 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl bromide for the synthesis of β-D-ribonucleosides has been carried out using 5,6-dimethylbenzimidazole as a base. While the desired β-D-ribonucleoside was obtained in 51% yield, its β anomer was isolated in 28% yield. From this finding, it is obvious that the presence of a participating group at C-2 in a glycosyl halide will not prevent the formation of substantial quantities of *cis* nucleosides. A procedure previously devised for the synthesis of the cytotoxic β-D-arabinofuranosyl nucleosides has been extended to the D-ribofuranose series. The condensation of 2,3,5-tri-*O*-benzyl-D-ribofuranosyl chloride with 5,6-dimethylbenzimidazole leads to the isolation of 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole (a moiety of vitamin B_{12}) in high yield (J. D. Stevens and R. K. Ness).

Synthesis of Specifically Blocked Ribonucleosides

While many ingenious methods have been devised for the specific blocking of hydroxyl groups in the sugar moiety of ribonucleosides, these often leave much to be desired. For the synthesis of ribonucleosides masked at C-2 with one type of substituent and at C-3 and C-5 with another type, it has now been found practicable to introduce the substituents into the sugar moiety prior to the synthesis of the nucleoside. For this purpose 3,5-di-*O*-benzyl-D-ribofuranosyl chloride and its 2-*O*-benzoyl and 2-*O*-*p*-phenylazobenzoyl derivatives have been found suitable. The last-named halide is particularly advantageous, since it leads to the formation of blocked nucleosides with visible color and these are readily purified by chromatography (M. Haga and R. K. Ness).

Selective Cleavage of the Glycosidic Bond in Acetylated 2-Acetamido-2-deoxy-β-D-glucopyranosides by a Chemical Transglycosylation

Conditions have been found under which acetylated 2-acetamido-2-deoxy-β-D-glucopyranose derivatives are converted into benzyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside. The corresponding α derivatives and other sugar derivatives (such as methyl β-D-glucopyranoside tetraacetate) are not attacked. The potential utility of this transglycosylation for the investigation of oligosaccharide structure was demonstrated with chitobiose octaacetate. The intersaccharidic linkage of this disaccharide was cleaved although, under the conditions employed, the major product was a mixture of the acetylated anomeric benzyl glycosides of chitobiose. Aside from its analytical aspects, the method is obviously applicable to the synthesis of *trans*-glycosides of the 2-acylamino-2-deoxyaldoses (W. L. Salo).

Synthesis of D-Xylo-Hexos-5-ulose 6-Phosphate, a Putative Intermediate in the Biosynthesis of myo-Inositol

The biochemical synthesis of *myo*-inositol from D-glucose 6-phosphate is NAD-dependent and it has been suggested that D-*xylo*-hexos-5-ulose 6-phosphate (5-ketoglucose 6-phosphate) is the intermediate at the actual cyclization step. In order to allow a test of this hypotheses, the presumed intermediate was prepared from D-glucose through a ten-step synthesis; samples are currently under biochemical investigation by Dr. F. Eisenberg, Jr. (LBM) (D. E. Kiely).

Syntheses with Partially Benzylated Sugars

Our knowledge of the ketoses is much less extensive than that of the aldoses and one reason for this is the lack of a general and practicable method for the synthesis of ketoses. It has now been shown that aldoses, fully benzylated save at carbon one and at the other carbon bearing the lactol ring, may readily be converted to ketoses in which the molecule is turned end for end, the atom originally bearing the lactol ring becoming the carbonyl group of the ketose. By way of illustration, the commercially available 2,3,5-tri-O-benzyl-D-arabinofuranose was converted into D-xylulose. The method would appear to be suited to the synthesis of otherwise inaccessible ketoses and, particularly those with a specific label or substituent (Y. Rabinsohn).

The Chemistry of Ketoses

In addition to the new method for the synthesis of ketoses described above, a general program, designed to broaden our understanding of this biochemically important class of substances has been initiated. In view of the great synthetic utility of the long-known, aldose-related glycals we have synthesized 1,4,5-tri-O-benzoyl-2,3-dideoxy-D-erythro-hex-2-enulopyranose, the first known ketose-related glycal with an endocyclic double bond (R. K. Ness).

Higher-Carbon Sugars, Anhydro Sugars, Sugar Alcohols, Disaccharides, and Their Derivatives

A new ketose-containing disaccharide, primeverulose (6-O-β-D-xylopyranosyl-D-fructose) has been synthesized through the alkaline rearrangement of primeverose (6-O-β-D-xylopyranosyl-D-glucose). The sugar will be used as a standard in examining plant extracts for the presence of primeverulose (N. K. Richtmyer).

Structures of the Antigen Occurring on Four Types of Dylococcus Pneumoniae Related by an Apparent Mutation

Serial subculturing of D. pneumoniae type 61 in the presence of cross reactive serum to type 31 causes mutation to a new type (M61) which has cross reactivity to serum against type 13. When this mutant, M61, is serially subcultured in the presence of serum to type 13, it reverts to the original parent type 61 (Laros). This is a unique system, and elucidation of 1) the structures of the antigens and 2) the capsule synthesizing enzyme systems, can shed real light on the cause for this mutation. A program has been undertaken to study the four types involved. Following the discovery of a facile method for the isolation of apparently pure capsular antigen from type 31, called S-31 (Glaudemans and Treffers, 1967), we have now shown that S-31 has a M.wt. of ca. 1.1×10^5 (sedimentation), and consists of glucuronic acid, galactose and rhamnose in the ratio of 1:2:2. The molecule appears mostly linear and it is likely that furanoside structures occur. The glucuronic acid is linked to galactose. Methylation studies are in progress, to elucidate the linkages (N. Roy and C. P. J. Glaudemans).

The Fine-Structure of Capsular Antigens from D. Pneumoniae and the Immunodominance of their Various Groupings

In S-34, the capsular polysaccharide of D. pneumoniae type 34, the O-acetyl group occurring on the internal galactofuranose residue appears to be on C-6. This follows from our findings that methyl 6-O-acetyl β-D-galactofuranoside, synthesized by two independent routes, is a good inhibitor of the S-34/rabbit anti S-34 precipitin system. The parent 6-hydroxyl compound does not inhibit.

We also prepared by synthesis 3-O-β-D-galactofuranosyl-D-glucose and its methyl α glycoside. This disaccharide occurs in type 34 pneumococcus. It is not a significant inhibitor, and hence this group is not immunodominant in S-34.

Three aldobiouronic acids were synthesized to test their inhibitory power on the S-II/anti II D. pneumoniae immune system: 6-O-α-D-glucuronosyl-D-glucose, 6-O-β-D-glucuronosyl-D-glucose and 4-O-α-D-glucuronosyl-D-glucose. The first one is a probable moiety of the antigenic polysaccharide of type II D. pneumoniae, and the inhibitory power of the three acids in the Type II/anti II immune system is presently being evaluated (N. Roy and C. P. J. Glaudemans).

SECTION ON MEDICINAL CHEMISTRY

Benzomorphan Studies

The synthesis of 6,7-benzomorphan, the parent structure of a relatively new class of efficacious analgesics developed in this laboratory, was finally achieved in 2% overall yield (10 steps from 4-phenylpyridine) after seven years of a sporadically continuing effort. It has been prepared recently, too, from pyridine itself in the usual Grewe synthesis. The key to this success was the use of polyphosphoric acid for the cyclization of the penultimate 2-benzyl-1-methyl-1,2,5,6-tetrahydropyridine instead of 85% H_3PO_4 or 48% HBr which simply added across the Δ^3-double bond. This parent structure with its secondary amine function and without a quaternary carbon, surprisingly, has appreciable analgesic activity, although it is rather toxic. The 2-methyl derivative is codeine-like, similar to the corresponding 5-methyl compound. These findings open new research avenues and should provide additional leads toward ultimate separation of deleterious from favorable CNS effects, already quite pronounced for this series (K. Kanematsu, A. E. Jacobson, J. H. Ager, M. Takeda and E. L. May).

O-alkyl, O-acyl and N-benzyl analogs of (±)-2,5-dimethyl-2′-hydroxy-6,7-benzomorphan, one of the simplest and most readily synthesized members of this series, have been prepared for testing as centrally acting analgesics and as antiinflammatory agents. Also the N-benzyl compound has been hydrogenolyzed to the secondary amine, not obtainable by the von Braun method or with diethyl azodicarboxylate. N- and O-glucuronides of this series are desired for biologic evaluation and metabolic studies (J. H. Ager).

The separation of (±)-6,7-benzomorphans into their optical isomers has been continued. The *levo*-rotatory isomers of 5-methyl- and 5-ethyl-2′-hydroxy-2-methyl-6,7-benzomorphans are, like those of the α-5,9-diethyl- and α-5-propyl-9-methyl-homologs, not only strong analgesics, but are also morphine antagonists (in monkeys) which will not support an established dependence upon morphine, but precipitate or exacerbate abstinence signs. All of the very weakly analgesic *dextro* compounds are, however, codeine- or morphine-like in physical dependence capacity. These are properties unique and consistent for this family of compounds and their discovery may set the stage for the development of the "ideal" analgesic agent. (J. H. Ager, A. E. Jacobson, and E. L. May).

Transformations in the Morphine Series

A compound, prepared in an intricate multistep sequence from thebaine, reported last year to be 2,3,4,5,6,6a-hexahydro-8-methoxy-3,11-dimethyl-1H-4,11b-methanobenzofuro[3,2-*d*]azocine (I), has been found to be instead, 1-methyl-3a-(3′-hydroxy-6′-methylphenyl-4,2′-oxidooctahydroindole (II) after comparison with a racemic degradation product of galanthamine and by an alternative synthesis. Product II which had no analgesic activity but which is optically active, was formed *via* rearrangement of an unexpected aziridinium intermediate in the original synthesis which was expected to give I. Compound I has now been synthesized by an unambiguous route. It is half as active as codeine, analgesically (M. Mokotoff and L. J. Sargent).

5-Methyldihydromorphinone (metopon) and dihydrodesoxymorphine-D (desomorphine) have been N-demethylated (these nor compounds have strong analgesic potency) and the nitrogen substituted with phenethyl, cyclopropylmethyl, and 3,3-dimethylallyl. The last two groups were chosen, because they should confer antagonistic properties and reduce or abolish dependence liability of the morphine type. The N-substituted compounds have good analgesic activity and will be assessed for dependence liability and antagonistic activity (L. J. Sargent and E. L. May).

Aldolase Inhibitors

After the trichloroacetonitrile method of phosphorylation failed, a direct method was developed which has given a 34% yield of *trans*-1,4-cyclohexanedimethanol monophosphate (I) and 24% of the *cis*- (II) monophosphate. So far, thermal degradation of the monophosphates has precluded preparation of the diphosphates, although it is now believed that this problem can be circumvented by the use of infrared radiation (to increase the population of transition-state intermediates) which has already given a 17% greater yield of monophosphate than the thermal method. The rate by the photochemical method is 4 times that of the thermal reaction. The monophosphates (I and II) are to be studied biologically as inhibitors of glycolytic enzymes. As the binding energy of hexose phosphate to aldolase involves primarily the anionic centers of the substrate (Hartman and Baker, 1965), the above phosphate esters may compete with hexose phosphates for this and possibly other enzymes (J. G. Murphy).

Evaluation of Analgesics

Dose-range-finding experiments were performed on 90 compounds, all of which were new, several from the Section on Medicinal Chemistry. Of these, 80 were subjected to complete assay (mainly by the subcutaneous route), 24-hour toxicities were determined for five for a total of 295 experiments. The more interesting compounds were sent to the University of Michigan for dependence liability determinations. Technical assistance was also provided Drs. Daly and Creveling (Metabolites) in their studies on biogenic amines (L. Atwell, E. L. May, and N. B. Eddy).

Instrumentation

A sensitive, solid-state electrometer for use in gas chromatography, (detection sensitivity 10^{-15} mole of carbon) has been developed. It has been found that copper, monel, and inconel columns are superior to brass and particularly any columns lined with teflon for steroids and steroid alkaloids.

A satisfactory timing and readout system has been developed for precision viscometry (T. D. Perrine with I. Tice, and H. Saroff).

Antigenic Polymers

When highly purified p-acetoxystyrene, maleic anhydride, and vinylpyrrolidone are copolymerized and the product subsequently treated with 2-dimethylaminoethylamine, an amphoteric material containing lactam, amide, carboxyl and phenolic hydroxyl groups is obtained. This material readily couples with diazonium compounds, is water soluble in all pH ranges and is reversibly denatured by drying. Attempts to form the glucoside of this ter polymer (which will be tested for antigenic and interferon-producing properties) have been unsuccessful. Coupling of the polymer with diazotized arsanilic acid gives red, water-soluble products which are being examined for antigenicity.

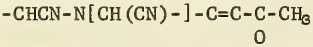
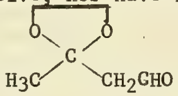
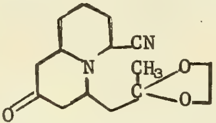
Amino acid-grafted polyethylenimines PEI (Leuch's anhydride types prepared from α-azido β-phenylpropionyl chloride and PEI in pyridine) are being examined as stimulators of interferon production and for copper chelation in vivo, in relation to collagen insolubilization studies.

Further routes to the monomer 4-p-(hydroxyphenyl)-1-vinyl-2-pyrrolidinone desired for homo- and Co-polymerization have been explored (T. D. Perrine and W. R. Landis).

Immunologic Studies in Clinical Hematology

Dihydroquinine (from the catalytic reduction of quinine) was O-demethylated to dihydrocupreine and the resulting phenolic base converted to 6-aminodehydrocinchonidine (NH_4HSO_3 sealed tube). The latter was coupled (after diazotization) with normal human and with certain animal sera, and the diazoproteins thus formed were examined for antigenic activity (L. J. Sargent and N. R. Shulman, CH).

Development of Synthetic Procedures for Alkaloids

Attempts to develop an annelation method for the cycl[3.3.3]azine system have been focused on 2,6-dicyanopiperidine after an initial success in displacing one of the nitrile groups with acetone. Similar efforts to introduce acetone into the  $-CHCN-N[CH(CN)-]-C=C-\underset{O}{C}-CH_3$ (I) system have so far been negative, nor have imino derivatives of I proved useful. At present the aldehyde  which should, under Mannich conditions, readily give  is being investigated. This research is being pursued with the aim of extending ideas of synthetic limitations in this area (E. M. Fry).

Antiinflammatory Activity

When evaluated in the Carrageenin-Induced Abscess test (rats), several 6,7-benzomorphans, all but one of a low order of central analgesic activity, showed significant reduction in abscess weight when compared with phenylbutazone-treated rats and controls. One of these, α-2′-fluoro-2,5,9-trimethyl-6,7-benzomorphan showed a good dose-response relationship. Bis-(cis-2,5-dimethyl-1-formyl-4-piperazinyl)methane also caused some reduction in abscess weight. A 1-phenylpropyltetrahydroisoquinolino compound derived from narcotine and previously found active in the UV erythema test proved to be aspirin-like in the Carrageenin system.

In addition five dihydro-1,3-oxazines derived from salicylic and 1-hydroxy-2-naphthalene carboxylic acids were more or less effective in the Croton Oil-Granuloma technique. 3-Cyclohexyl-2-cyclohexylimino-3,4-dihydro-2H-1,3-benzoxazine caused 55% inhibition of inflammation at 5 mg/kg (rat). Hydroxycortisone acetate produced 90% inhibition at a comparable dose (A. E. Jacobson and E. L. May).

SECTION ON METABOLITES

The Structure of Batrachotoxinin A, a Novel Steroidal Alkaloid from the Colombian Arrow Poison Frog, *Phyllobates aurotaenia*

Earlier studies on the potent venom from the Colombian arrow poison frog had indicated the presence of three major alkaloids, batrachotoxin, batrachotoxinin A, and batrachotoxinin B. Batrachotoxin, the most active cardiotoxin known (ED_{50}, intravenous in anesthetized dogs, <0.5 μg/kg), was the major steroid alkaloid isolated from these frogs.

When the earlier isolation (distribution between two solvents, preparative tlc) procedures were modified and all operations carried out at 5°, very little batrachotoxinin A was present after the initial purification. The major active fraction has now been resolved into two very similar isomers of almost equal toxicity, viz., batrachotoxin (LD_{50}, subcutaneous in mice, 2 μg/kg) and isobatrachotoxin (LD_{50}, 3 μg/kg). In addition, a third component, pseudobatrachotoxin, is present in the extracts and cochromatographs with isobatrachotoxin. It is very unstable and readily converted to batrachotoxinin A, formally by the addition of the elements of water. Thus, batrachotoxinin A is probably a secondary product formed from the highly unstable pseudobatrachotoxin during purification. Batrachotoxinin A (LD_{50}, 1 mg/kg), which retains only 1/500 of the toxicity of the original venom, is still almost as toxic as strychnine (LD_{50}, 0.5 mg/kg).

Pure batrachotoxin, isobatrachotoxin, and batrachotoxinin A have now been isolated by column chromatography on silica gel (20 W) and by elution with a mixture of cyclohexane, chloroform, triethylamine, and methanol (16:4:1). If one assumes that all the batrachotoxinin A arises from pseudobatrachotoxin, then batrachotoxin, isobatrachotoxin, and the sum of pseudobatrachotoxin and batrachotoxinin A occur in fresh extracts in the ratio of 3:1:5. The fragmentation pattern of the high-resolution mass spectrum (AEI MS-9 mass spectrometer, direct inlet 240°) indicates the close relationship of the three isomers ($C_{24}H_{33}NO_4$). Infrared spectra of batrachotoxin and isobatrachotoxin show an intense absorption band at 1690 cm^{-1} which is missing in batrachotoxinin A. The band previously reported at 1645 cm^{-1} for batrachotoxinin A is due to an impurity. The ultraviolet spectra of batrachotoxin and isobatrachotoxin are virtually identical and have characteristic maxima at 234 mμ (ε 9200) and 264 mμ (ε 5100). Batrachotoxinin A and pseudobatrachotoxin have only end absorption as judged from direct measurement and from difference spectra of isobatrachotoxin compared with mixtures of pseudobatrachotoxin and isobatrachotoxin. The previous assumption of only end absorption for batrachotoxin rested on the low extinction coefficients now considered to be caused by the presence of pseudobatrachotoxin.

Batrachotoxinin A ($C_{24}H_{35}NO_5$) has now been acylated with *p*-bromobenzoic anhydride under Schotten-Baumann conditions to an O-*p*-bromobenzoate. This product was purified by column chromatography on silica gel (10 W) with chloroform containing 3% methanol and recrystallized from acetone to yield fine needles (m.p. 213°). Mass spectrometry established the composition as $C_{31}H_{38}NO_6Br$. Saponification of this *p*-bromobenzoate with 0.05 N alkali at room temperature gave back batrachotoxinin A and not a new or rearranged product.

An X-ray diffraction analysis of a single crystal of this derivative was made by Mrs. I. Karle, U. S. Naval Research Laboratory, using three-dimensional intensity data which were collected using CuKα radiation and the equiinclination, multiple-film Weissenberg technique. It was possible to record only a total of 830 independent reflections from a crystal 0.05 x 0.03 mm in cross section. The material crystallizes in the orthorhombic system, space group $P2_12_12_1$ with four molecules in the unit cell and cell dimensions $a = 15.42 \pm 0.03$ Å, $b = 7.05 \pm 0.02$ Å, and $c = 26.50 \pm 0.04$ Å. The Br atom was readily located from a Patterson map to be at 1/5,0,0 and phases based on the position of the Br atom alone gave rise to an electron density map with a fourfold ambiguity for the remainder of the structure. It was possible to identify the two oxygen atoms for the p-bromobenzoate group in this map, and thus locate approximately the p-bromobenzoate group in the unit cell. Using this group as a known partial structure, phases were obtained for some of the strongest reflections. These phases were used with the tangent formula in recycling procedure to obtain additional phases. Further atoms were found in the resulting E maps. In several cycles all 39 atoms were located. E maps computed from phases obtained in this manner are much better resolved than those obtained by the heavy atom method. In this particular case, the fact that the heavy atom was specially placed and the data were severely limited made the direct application of the heavy atom method quite unsuitable.

The identification of the six oxygen atoms was obvious from the weights of the peaks in the E maps. The identification of the nitrogen atom was based on the size of the thermal factors in the least-squares refinement, difference maps, and bond lengths. A least-squares refinement of the coordinates for each atom, isotropic thermal factors for all atoms except for the Br atom, and anisotropic thermal factors for the Br atom resulted in an agreement factor of 9.7%.

The structure of batrachotoxinin A is 3α,9α-epoxy-14β,18β-[epoxyethano-N-methylimino]-5β-pregna-7,16-diene-3β,11α,20α-triol.

Toxicity of Panamanian Poison Frogs (Dendrobates): Some Biological and Chemical Aspects

A small Neotropical frog, Dendrobates pumilio, undergoes interpopulational variation in color, degree of toxicity, size, and habits. Differences in body coloration encompass the visible spectrum from red to blue, as well as achromatic black and white. There are wide variations in the degree of toxicity, but these variations are not correlated with supposed warning colors. Extracts of skin yield two toxic compounds characterized as steroidal alkaloids with molecular formulae $C_{19}H_{33}NO_2$ and $C_{19}H_{33}NO_3$. The rapid rate of divergent evolution among populations of this frog may result from isolation and chance restriction of original heterozygosity, with subsequent selection acting on different and greatly limited mixtures of alleles (J. W. Daly and C. W. Myers).

Venoms of Other Amphibians

Examination of 7 species of Puerto Rican frogs of the related genus Eleutherodactylus did not reveal significant quantities of any biogenic amines such as tyramine, candicine and leptodactyline, and the imidazole-alkylamines, such as histamine and spinceamine. A large amount (0.2-1.2 mg/g skin) of an amino acid which gave a Pauli positive color reaction typical of an imidazole was found in all these frogs (J. W. Daly and H. Heathwole, in press). This amino acid was not histidine and was at first thought to be spinacine, the amino acid precursor of the amine spinceamine.

Chemical studies proved, however, that the material was the dipeptide, carnosine (β-alanylhistidine) previously known only from the muscle of certain vertebrates. Carnosine has no known physiological or pharmacological activity and certainly is not present as a protective poison in these frogs.

Other peptides of higher molecular weight have been found in a variety of amphibians: Bradykinin and physalaemin, are potent hypotensive agents, while the tryptokinins have no known activity (Daly and Witkop).

The Nonenzymatic Conversion of Tyrosine into Mono- and Dihydroxyindoles

The reaction of methyl or ethyl tyrosinate, tyrosineamide, and N-methylamide in dilute aqueous acetic acid with N-bromosuccinimide (NBS) or bromine results first in rapid o,o'-bromination and a commensurate increase of the absorption of the phenolic chromophore at 275-280 mμ. A third mole of NBS produces the labile tribromodienone (λ_{max} 270 mμ) which can either revert to the dibromotyrosine level or rearrange to a 5,7-dibromo-6-hydroxyindole resulting from intramolecular Michael addition of the amino group of the tyrosine side chain to the dienone system. At pH 6 this rearrangement proceeds with loss of two bromines to a 5,6-dihydroxy-7-bromoindole which was obtained also by the action of NBS on the ethyl ester of 3,4-dihydroxyphenylalanine (dopa) with ethyl 5,6-dihydroxy-7-bromo-2,3-dihydroindole-2-carboxylate (isolated as the 5,6-diacetate) as an intermediate or by-product which is spontaneously dehydrogenated to the corresponding indole on standing in alkaline solution. Oxidation of dopa ethyl ester by NBS in addition gave a bromine-free product which was isolated as the O,O,N-triacetate (M. Wilchek, T. Spande, G. W. A. Milne, and B. Witkop).

A Convenient Spectrophotometric Procedure for the Determination of Amino-Terminal Tyrosine Residues

The conversion of NH_2-terminal tyrosine residues with 4 equivalents of NBS in acetic acid buffer, or with excess NBS in formic acid, into the chromophore of 5,7-dibromo-6-hydroxyindole-2-carboxamide (λ_{max} 315 mμ) permits the rapid assay of NH_2-terminal tyrosine in model peptides and tryptic fragments from glucagon, globin, and chymotrypsinogen, in which the sequence -Arg-Tyr or Lys-Tyr- occurs with more than random frequency. After tryptic digestion, glucagon showed one, globin two, and chymotrypsinogen A three NH_2-terminal tyrosine residues per mole by this method (M. Wilchek, T. Spande, and B. Witkop).

Photocyclizations of Tyrosines, Tyramines, Catecholamines, and Normescaline

N-Chloroacetyl-*m*-tyramine and -*m*-tyrosine on irradiation in aqueous solution are photocyclized to 7-hydroxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one and its 4-carboxy derivative which are methylated to the 7-methoxy- and 7-methoxy-4-carbomethoxy derivative. The 7-methoxy lactam is reduced to 7-methoxy-1,2,4,5-tetrahydro-3H-3-benzazepine by diborane in tetrahydrofuran. N-Chloroacetyl-*p*-O-methyltyramine and -*p*-O-methyl-L-tyrosine on irradiation in aqueous solution undergo a deep-seated photolytic rearrangement to yield 7-formyl-1,2,5,6-tetrahydro-3H-pyrrolo[1,2-*a*]azepin-3-one and L-5-carboxy-7-formyl-1,2,5,6-tetrahydro-3H-pyrrolo[1,2-*a*]azepin-3-one, the structure of which, an asymmetric molecule, was determined by X-ray crystallography of the methyl ester without the benefit of a heavy atom. N-Chloroacetyl-3,4-dihydroxyphenethylamine undergoes photocyclization with ring closure into the two positions *para* and *ortho* to the *m*-hydroxy group. After acetylation the crystalline photocyclization products were N-acetyl-7,8-diacetoxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one and N-acetyl-8,9-diacetoxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one. N-Chloroacetyl-3-hydroxy-4,5-dimethoxyphenethylamine, a derivative of normescaline, on irradiation gave a single product, the anhalonidine analog 7,8-dimethoxy-9-hydroxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one, resulting from photocyclization into the position *ortho* the the phenolic hydroxyl. N-Chloroacetyl-, N-bromoacetyl-, and N-iodoacetylmescalines in preliminary photocyclization attempts were converted to N-acetylmescaline (O. Yonemitsu, T. Tokuyama, M. Chaykovsky, and B. Witkop).

Photocyclizations of N-Chloroacetyl-3,4-dimethoxyphenethylamine

The photolysis of N-chloroacetyl derivatives of aromatic amino acids and pharmacodynamic amines so far has led to tricyclic indoles, benzazepines, and aza-azulenes.

We have now found two novel photocyclizations of N-chloroacetyl-3,4-dimethoxyphenethylamine. The parent amine has been isolated by members of the National Institute of Arthritis and Metabolic Diseases as a constituent of human (schizophrenic?) urine (C. R. Creveling and J. W. Daly, *Nature*, **216**, 190 (1967)). On irradiation *in ethanol-water solution* with a 100-watt mercury lamp for 1.5 hr at room temperature, the cyclization reaction, in analogy to that of N-chloroacetyldopamine, yielded the two expected benzazepines.

In addition a deepseated photorearrangement gave 11% of a tetracyclic product.

On the other hand, irradiation of the amine *in tetrahydrofuran solution* under the conditions described above gave a novel ten-membered lactam, 12-methoxy-2-oxa-6-aza-bicyclo[7.3.1]trideca-1(13),9,11-triene-5-one, as the major product which on heating in 48% hydrobromic acid at 140° for 2 hr., was converted to the novel tricyclic 9-hydroxy-2,3,5,6-tetrahydropyrano[2,3,4-ij]isoquinoline hydrobromide.

There is precedent for the participation of an aromatic methoxy substituent in the photocyclization of quercetin pentamethyl ether. The $-OCH_2^{\bullet}$ radical may be formed by abstraction of $H^{\bullet}$ through $Cl^{\bullet}$ generated in the photo-induced homolysis of the C-Cl bond which is known to depend on the nature of the solvent. The results presented here point to two simultaneous primary reactions. The n,π*-activation of the carbonyl group of N-chloroacetyl-3,4-dimethoxyphenethylamine may lead to elimination of the α-substituent, viz., $Cl^{\ominus}$, as an anion and a positive fragment which is capable of electrophilic aromatic substitution. In non-polar aprotic solvents a radical mechanism would favor formation of the 10-membered lactam. Such a dualism of photolysis has recently been observed for α-sulfonyloxyketones (O. Yonemitsu, Y. Okuno, Y. Kanaoka, and B. Witkop).

The Photolysis of N-Chloroacetyl-3,4-dimethoxyphenethylamine to DL-1,2,5a,7b-Tetrahydro-5a,5b-dimethoxy-5bH-cyclobuta[1,4]cyclobuta[1,2,3-gh]pyrrolizin-4(5H)-one, X-Ray Analysis and Proof of Structure

Irradiation of N-chloroacetyl-3,4-dimethoxyphenethylamine both in aqueous ethanol and in tetrahydrofuran produced a photocyclization product, mp 123.5°, in 10-12% yield, for which the structure of 1,2,5a,7bβ-tetrahydro-5aβ,5bα-dimethoxy-5bH-cyclobuta[1,4]-cyclobuta[1,2,3-gh]pyrrolizin-4(5H)-one, has been proven.

An X-ray diffraction analysis of a single crystal of $C_{12}H_{15}NO_3$ has established the structural formula and the configuration of the photoproduct. Eleven hundred independent reflections were recorded with Cu radiation by the multiple-film, equiinclination Weissenberg technique. The space group is $P2_12_12_1$ with four molecules per unit cell and the cell parameters are a = 11.75 A, b = 6.27 A and c = 14.70 A. Phases for the strong and moderately strong reflections were determined directly from the experimental intensities by the symbolic addition procedure for noncentrosymmetric crystals. In the initial density map computed with 306 reflections whose phases had been determined, the positions of the thirteen strongest peaks suggested an unusual arrangement of atoms. The coordinates of the thirteen peaks were used as a partial structure in a recycling procedure employing the tangent formula and the remaining three carbon atoms were located. Hydrogen atoms were found in a difference map. A least-squares refinement of the coordinates and thermal parameters has reached an agreement factor of 10.0%. The stability of such a highly strained policyclic molecule to X-ray radiation over a total of 300 hours is remarkable (O. Yonemitsu, Y. Okuno, Y. Kanaoka, I. L. Karle, and B. Witkop).

Deuterium Migration during the Acid-Catalyzed Dehydration of 6-Deuterio-5,6-dihydroxy-3-chloro-1,3-cyclohexadiene, a Nonenzymatic Model for the NIH Shift

In most enzymatic hydroxylations of aromatic substrates the substituent (^2H, ^3H, or halogen) present at the position of the entering oxygen migrates to either one of the adjacent ring positions. This migration has been called the NIH Shift. In a variety of chemical hydroxylating systems only one reagent, trifluoroperacetic acid, produced these hydroxylation-induced migrations. The electrophilic nature of trifluoroperacetic acid suggests cationoid intermediates. A general mechanism involving such cationoid intermediates would conveniently rationalize the migration observed both in nonenzymatic and enzymatic hydroxylation reactions. Enzymatic hydroxylations with concomitant migration and retention of isotopic label proceed *via* cationoid intermediates. This has been proved by starting with 6-deuterio-5,6-dihydroxy-3-chloro-1,3-cyclohexadiene isolated from the urine of rabbits to which 4-deuteriochlorobenzene had been administered.

About 1 mg of the glycol was dehydrated with 1.0 N HCl at 100° for 10 min. The phenolic products were methylated with dimethyl sulfate in oxygen-free 1.0 N NaOH at 80° for 2-3 min, conditions under which no appreciable exchange occurs. The resulting chloroanisoles were examined by combined glpc-mass spectrometry with Carbowax columns operating at 115-130°. The mixture consisted of 99% 4- and 1% 3-chloroanisole. *The 4-chloroanisole contained 0.23, and the 3-chloroanisole approximately 0.84 deuterium atom per molecule.* When the dehydration was conducted at room temperature in 1.0 N HCl for 4 days, the isolated *p*-chloroanisole contained 0.19 deuterium atom per molecule. The retention of 19-23% deuterium in the 4-chlorophenol by acid-catalyzed dehydration of the glycol compares with 54% retention *in vivo* where the phenol is probably formed directly rather than via the glycol.

The results allow two conclusions: (i) retention of deuterium in the dehydration of the glycol involving cationic intermediates is significant; (ii) Migration and loss of hydrogen by an enzymatic pathway leads to 3-chlorophenol with retention of 84% ^{2}H. The 16% loss of deuterium in this instance provides the first evidence for the NIH shift to hydrogen itself. The retention of deuterium (tritium) in metabolites of selectively labeled aromatic substrates will permit a more detailed understanding of the multiple pathways and interrelationships in the breakdown of physiological and pharmacological agents *in vitro* and *in vivo* (D. M. Jerina, J. W. Daly, and B. Witkop).

Hydroxylation of Alkyl and Halogen-Substituted Anilines and Acetanilides by Microsomal Hydroxylases

The hydroxylation of a variety of halo and alkyl-substituted anilines and acetanilides with hepatic microsomal preparations was studied. 4-Chloro- and 4-fluoroacetanilides and anilines were hydroxylated to the corresponding 4-hydroxy derivatives with loss of halogen. The 4-chloro derivatives were extremely poor substrates for the aryl hydroxylases. 3-Methyl-, 3-chloro-, and 3-fluoroacetanilides were metabolized to the corresponding 4-hydroxy-3-substituted acetanilide. 4-Methylaniline, 4-methylacetanilide, and 4-ethylacetanilide were not substrates for aryl hydroxylation, but instead underwent side-chain oxidation to form, respectively, 4-hydroxymethylaniline, 4-hydroxymethylacetanilide, and 4-(1'-hydroxyethyl)acetanilide. The hydroxymethyl compounds were further oxidized to aldehydes (J. W. Daly, G. Guroff, S. Udenfriend, and B. Witkop).

Enzymic and Nonenzymic Hydroxylation and Chlorination of p-Deuteroanisole

The discovery of the hydroxylation-induced migrations of substituents during enzymic hydroxylation of aromatic rings has permitted new insights into the mechanism of enzymic aryl hydroxylation. The deuterium and tritium retentions in a variety of substrates have been correlated with the influence of the various substituents on charge distribution in the proposed cationoid intermediates. The mechanism proposed for the migration requires a hydroxyl group for the stabilization of the rearranged product as the cyclohexadienone. On this basis one would predict that other enzymic or chemical substitutions of aromatic rings, such as chlorination, which could not lead to cyclohexadienone type stabilization, would lead to loss rather than migration of substituents. Confirmation of this expectation was found in the essentially complete loss of deuterium from p-deuteroanisole on either enzymic or nonenzymic chlorinations in the *para* position.

Hydroxylation of the same substrate with microsomal preparations produced p-hydroxyanisole with 60% of the initial deuterium via a migration and retention pathway. This retention is higher in relationship to migration with other substrates than would be predicted on the basis of delocalization of charge in cationoid intermediates by electron-donating substituents (D. Jerina, G. Guroff, and J. Daly).

Intramolecular Migration of Tritium and Deuterium during Nonenzymatic Aromatic Hydroxylation

The nature of the oxygenating species involved in the enzymatic hydroxylation of aromatic substrates is still a subject for speculation. Of the many hydroxylating systems which have served as models for hydroxylases only trifluoroperacetic acid showed retention of tritium, deuterium and even halogen. Retentions up to 75% in the case of p-deutero-halobenzenes have now been observed. The degree of retention in this and other model systems currently under investigation may serve as a useful further guide for the correlation of enzymatic and nonenzymatic hydroxylations (D. Jerina, J. Daly, W. Landis, and B. Witkop).

Quantitative Studies on the Hydroxylation-Induced Migration of Deuterium and Tritium During Phenylalanine Hydroxylation

Quantitative studies on the hydroxylation of deuterated and tritiated phenylalanines by phenylalanine hydroxylase have been carried out. Over 90% of the 4-tritium of phenylalanine migrates upon hydroxylation and is retained in the 3-position of the product, tyrosine. No isotope effect is involved in the hydroxylation of 4-deutero- or 4-tritiophenylalanine, or of 3,5-dideuterophenylalanine. The release of tritium from the 3-position during 4-hydroxylation was studied. The small amount of release observed provides evidence as to the mechanism of the hydroxylation-induced migration (G. Guroff and J. Daly).

Intramolecular Migration of Deuterium and Tritium during Enzymic Hydroxylation of *p*-Deuteroacetanilide and *p*-Tritioacetanilide

The hydroxylation of *p*-tritio- or *p*-deuteroacetanilide with the aryl hydroxylase of rabbit liver microsomes leads to the production of *m*-tritio- and *m*-deutero-*p*-hydroxyacetanilide, respectively. Studies with the tritiated material show that 45-50% of the tritium is retained in the product. The radioactivity of the substrate is not randomized or exchanged prior to hydroxylation. The radioactivity of the product is not lost upon further incubation with the enzyme. The tritium not retained in the product can be found as tritiated water. Initial experiments show that the amount of retention can be varied by varying the pH of the incubation. Nonenzymic hydroxylation with a modified Fenton's reagent led to complete removal of the *p*-tritium as tritiated water. These and other data indicate that enzyme-catalyzed aromatic hydroxylation is accompanied by a stepwise intramolecular migration of the group displaced by hydroxyl. A mechanism for this migration is presented and discussed (S. Udenfriend, P. Zaltzman-Nirenberg, J. Daly, G. Guroff, C. Chidsey, and B. Witkop).

Hydroxylation-Induced Migrations of Tritium in Several Substrates of Liver Aryl Hydroxylases

The hydroxylation of a variety of specifically tritiated aromatic substrates of liver aryl hydroxylases has been studied to help elucidate the mechanism of the "NIH Shift." Oxidation of phenols, e.g., of 5-tritiosalicylic acid to gentisic acid, or of N-acetyl-3,5-ditritiotyramine to N-acetyl-5-tritiodopamine, does not lead to significant migration and retention of tritium label as a result of hydroxylation. The conversion of 4-tritioacetanilide to 4-hydroxyacetanilide *in vivo* in rats or *in vitro* with rabbit or rat microsomes is accompanied by migration and retention of 38-56% of the tritium label. Hydroxylation of 4-tritioaniline to 4-hydroxyaniline proceeds with 12% retention. *In vivo* (rat) 4-tritioamphetamine is metabolized to *p*-hydroxyamphetamine with 91% retention. The results are discussed in terms of possible cationoid intermediates which would lead to the observed migrations and retentions (J. Daly, G. Guroff, S. Udenfriend, and B. Witkop).

Isotope Studies on the Mechanism of Action of Adrenal Tyrosine Hydroxylase

The position of ^{18}O in the dopa formed from either tyrosine-4-^{18}O and $^{16}O_2$ or from nonisotopic tyrosine and $^{18}O_2$ by tyrosine hydroxylase has been determined. The methodology included the enzymatic conversion of dopa to 3-methoxytyramine and the periodate oxidation of this material to the quinone and methanol. The ^{18}O content of the methanol was determined by mass spectrometry. Based on this assay, the dopa formed from tyrosine-4-^{18}O contained only small amounts ($\sim$10%) of ^{18}O in the 3-position while dopa formed from nonisotopic tyrosine and $^{18}O_2$ contained approximately 90% of the label in the 3-position. Hydroxylation of phenylalanine-4-^{3}H with tyrosine hydroxylase resulted in migration of the tritium from the *para*-position since the final product, dopa, contained 42% of the original radioactivity (J. Daly, M. Levitt, G. Guroff, and S. Udenfriend).

Phenol-O-Methyltransferase

An enzyme that transfers the methyl group of S-adenosylmethionine to phenol to form anisole has been characterized. The enzyme is highly localized in the microsomes of the liver and lung of mammals and is also present in other tissues. A variety of simple alkyl-, methoxy-, and halophenols can serve as substrates for the phenol-O-methylating enzyme. Activity of the enzyme is inhibited by sulfhydryl reagents and SKF 525. Hypophysectomy in rats increases the activity of phenol-O-methyltransferase in rat liver but not in lung or brain (J. Axelrod and J. Daly).

Latent Derivatives of Norepinephrine

The synthesis of the methoxymethyl ethers of phenolic compounds such as testosterone, morphine and phenazocine, and the carbonates of amines such as normeperidine represents approaches to the preparation of latent drugs. The use of triacetoxynorepinephrine and 3,4-carbonylnorepinephrine or related derivatives represents a feasible way of providing in the central nervous system a sustained release of the neurotransmittor, norepinephrine (J. Daly, C. Creveling, T. Tokuyama, and B. Witkop).

The Chemorelease of Norepinephrine from Mouse Hearts; 3,5-Dihydroxy-4-methoxyphenethylamines and Related Compounds

The effects of the amino acids corresponding to 3,5-dihydroxy-4-methoxyphenethylamine and 3,4,5-trihydroxyphenethylamine were studied. Both cause profound release of norepinephrine similar to that caused by α-methyl-*m*-tyrosine. The release by 3,5-dihydroxy-4-methoxyphenylalanine (50% of control at 31 mg/kg) was blocked (76% of control) when the mice were pretreated with 50 mg/kg of the aromatic amino acid decarboxylase inhibitor, N-DL-seryl-2,3,4-trihydroxybenzylhydrazone indicating that the amino acid is first decarboxylated to the amine, which then causes the release of norepinephrine-^3H. The 3,5-dihydroxy-4-methoxyphenylalanine was tested without signs of toxicity at levels of 1000 mg/kg in mice. Two hours after the administration of this amino acid (50 mg/kg s.c.) the level of norepinephrine in brain had decreased to 60% of normal. The same dose depleted cardiac norepinephrine-$^3H^2$ to a level corresponding to 34% of control. The 3,4,5-trihydroxyphenylalanine depletes endogenous norepinephrine in heart, spleen and brain. Norepinephrine derivatives with methyl substituents in the ring *retained* appreciable activity which was found to be reduced by N-alkylation (C. Creveling, J. Daly and B. Witkop).

The Chemorelease of Norepinephrine from Mouse Hearts; Structure-Activity Relationships of Reserpines and Yohimbines

Forty-five congeners and stereoisomers of the hypotensive alkaloids, reserpine and yohimbine, have been tested for the release of tritiated norepinephrine in mouse hearts. Novel structure-activity relationships have been uncovered (C. Creveling, J. Daly, R. Parfitt, and B. Witkop).

The Depletion of Cardiac Norepinephrine by 3,5-Dihydroxy-4-methoxyphenethylamine and Related Compounds

A comparison was made of the ability of phenolic phenethylamines and their O-methyl congeners to deplete both prelabeled cardiac H^3-norepinephrine and endogenous norepinephrine stores in the mouse. O-Methyl derivatives were inactive with the notable exception of 3,5-dihydroxy-4-methoxyphenethylamine, which proved to be an effective releasing agent. The ED_{50} of this amine, the β-hydroxylated analog and 3,4,5-trihydroxyphenethylamine was determined and compared to reserpine, l-metaraminol, 6-OH dopamine and tyramine. The effects of cocaine and bretylium on the release of norepinephrine induced by these trioxy amines were determined. The metabolism of H^3-norepinephrine in the isolated perfused rat heart during release with 3,5-dihydroxy-4-methoxyphenethylamine was compared to the metabolism obtained with reserpine and tyramine perfusion. A shift toward metabolism by monoamine oxidase was observed and a marked increase in the release of free catecholamine. Evidence is presented showing that 3,5-dihydroxy-4-methoxyphenethylamine was taken up by the heart and converted to the β-hydroxylated analog *in vivo*; also, this amine was shown to be a substrate for dopamine-β-hydroxylase *in vitro* (C. Creveling, J. Daly, and B. Witkop).

Reduction of Tissue Norepinephrine and Blood Pressure by 3,5-Dihydroxy-4-methoxyphenylalanine

3,5-Dihydroxy-4-methoxyphenylalanine (DHMPA) has been shown to be an effective depletor of norepinephrine content of the heart, brain and spleen in mouse and rat. Significant antihypertensive effects of the drug were observed in spontaneously hypertensive rats. This action of the drug appears related to the catecholamine depletion and a possible false transmitter mechanism (S. Spector, R. Tabei, C. Creveling, J. Daly, B. Witkop, and A. Sjoerdsma).

Identification of 3,4-Dimethoxyphenethylamine from Schizophrenic Urine by Mass Spectrometry

Dansylation, thin-layer chromatography and mass spectrometry as a combined technique have made possible the identification of the so-called "pink-spot" in schizophrenic urine as 3,4-dimethoxyphenethylamine (C. Creveling and J. Daly).

Studies on the Biosynthetic and Catabolic Enzymes Concerned with Biogenic Amines

A modified method for the purification of catechol-O-methyltransferase has been developed. The purified enzyme has been used to develop a sensitive assay for nanogram quantities of norepinephrine. A sensitive enzyme assay, using dihydroxybenzoic acid as substrate, has been developed and used to study the species variation, anatomic and intracellular distribution and induction of this enzyme *in vivo* by such agents as phenobarbital and benzypyrene. A convenient, nonisotopic, spectrophotometric assay, using 4-nitrocatechol as substrate, has been developed. This assay has been used to study the kinetics of enzyme inhibition and to provide a simple screen for inhibitors of this enzyme.

The anatomic and intracellular distribution of tyrosine hydroxylase, dopa decarboxylase and dopamine-β-hydroxylase in the canine brain has been determined.

A microradiochemical assay for dopamine-β-hydroxylase has been developed. β,β-Dideuterotyramine and α,α-dideuterotyramine have been synthesized to confirm a primary isotope effect observed with the corresponding β,β-ditritio compound (C. Creveling, B. Nikodijevic, J. Daly, T. Kunieda, and D. Jerina).

Studies on the Mechanism of Enzymatic Hydroxylation

Further examples of group migrations for alkyl, chloro, bromo and trifluoromethyl substituents have been demonstrated. Thus, hydroxylation of 4-methylphenylalanine with phenylalanine hydroxylase gives rise to 3-methyltyrosine. Surprisingly, the other product formed with this enzyme is 4-hydroxymethylphenylalanine. This represents the first clear instance of an aliphatic hydroxylation catalyzed by an aryl hydroxylase, in this case phenylalanine hydroxylase.

Various arene oxides, often suggested as intermediates in aryl hydroxylation and in the formation of dihydrodiols and premercapturic acids, have been synthesized. These were benzene oxide, 4-deuterotoluene 3,4-oxide and napthalene oxide. Benzene oxide, in vitro with liver preparations, gives rise to phenol, benzenedihydrodiol and a premercapturic acid. The phenol is formed nonenzymatically while the other products are formed respectively by a specific epoxide hydrase and a glutathione-S-epoxide transferase. Direct evidence for the unstable benzene oxide as an intermediate could not, however, be obtained. The 4-deutero-toluene-3,4-oxide rearranges to 3-deutero-4-hydroxytoluene proving that arene oxides are also compatible with the "NIH Shift". Oxygen-18 studies with tyrosine hydroxylase are also compatible with the postulate of an intermediate 3,4-oxide (D. Jerina, J. Daly, and B. Witkop).

Alkylation of Tryptophan and Indole Derivatives with 2-Hydroxy-5-nitrobenzyl bromide ("Koshland's reagent")

This reagent, since its introduction in 1964 has been widely applied to label tryptophyl residues in protein studies. At least a dozen papers have appeared featuring this reagent, though the nature of the reaction product remains unknown. Employing the simple indole derivatives, skatole and 2,3-dimethyl indole, M. Wilchek and T. Spande were able to show that, with only a slight excess of reagent, in aqueous acetone, alkylation at the three-position in the pyrrole ring occurs to form initially an indolenine, which undergoes ring closure with the phenolic hydroxyl group originally on the reagent to form an indoline. The application of the same reaction to N-acetyltryptamine and N-acetyl-tryptophan ethyl ester led to the isolation of products which had incorporated two moles of reagent. A bis-labelled product was obtained homogeneous from N-Ac tryptamine in 60% yield when two equivalents of reagent were employed. A structure has been advanced based upon NMR, UV, IR and mass spectrometric studies (M. Wilchek, T. Spande, and B. Witkop).

The Alkaline Oxidation of N-Blocked, C-Blocked Tryptophyl Derivatives

The NBS oxidation of N-acetyl tryptophan esters or amides at pH 8-9 had previously (N. M. Green and B. Witkop: Trans. N. Y. Acad. of Sci. II, 26, p. 659, 1964) been observed to produce a strongly absorbing chromophore at 308 mμ. Isolation proved difficult, but yielded 30-40% of the material responsible for the chromophore by the use of a very dilute solution of the tryptophan derivative and the rapid addition of exactly one equivalent of NBS. The reaction was shown to be specific for derivatives of tryptophan with both amino and carboxyl blocked. The structure was proved unambiguously by 100 mc NMR and mass spectrometer studies for the oxidation product of N-acetyltryptophan ethyl ester.

A number of chemical reactions of this novel pyrrolo-indole have been studied including a very ready acid hydrolysis to an oxindole, the reduction of the indole double bond with hydrogen or deuterium, and acetylation. The same material can be much more conveniently prepared using t-butyl hypochlorite in a triethylamine buffered $CHCl_3$-CCl_4 solution. A second equivalent converts the indole derivative to a chloroindolenine. An acetoxy indolenine results with lead tetraacetate. Synthetic approaches to the metabolite, Sporidesmin-B, are currently being developed (M. Ohno, T. Spande, and B. Witkop).

2-Nitro-4-carboxy-benzenesulfenyl Chloride as a Useful Reagent for Visualizing and Isolating Tryptophyl Peptides on High-Voltage Paper Electrophoresis

The reaction of tryptophan with 2-nitrosulfenyl chloride has been shown by Scoffone et al., Biochem. Biophys. Res. Commun. 25: 170, 1966, to produce a 2-substituted aryl-thio indole. The reaction is quantitative and can be applied to assay tryptophan in proteins. Employing a reagent slightly modified with a carboxyl group would covalently bind a group to a tryptophyl residue which carries a potential negative charge. This should be useful for detecting and isolating tryptophan-containing peptides using the diagonal electrophoresis technique. Such a procedure would be particularly applicable to the rapid location of tryptophyl peptides in the course of modification studies (e.g. oxidation with NBS) on proteins. The synthetic precursor of the sulfenyl chloride reagent, a disulfide should be useful as a modified Ellman reagent for the detection of thiol groups in proteins (M. Ohno, T. Spande, and B. Witkop).

The Stereochemistry of the Catalytic and Light-Induced Reduction of Thymidine to Dihydrothymidine and Ureido Alcohols

Only one diastereoisomer of 1-dihydrothymidine was obtained by catalytic reduction of thymidine. This 1-dihydrothymidine on acid hydrolysis yielded (S)-(—)-dihydrothymine which was hydrolyzed to (S)-(+)-β-ureidoisobutyric acid, the antipode of the analogous thymidine metabolite (R)-(—)-β-aminoisobutyric acid. (—)-Dihydrothymidine was hydrogenolyzed by sodium borohydride in aqueous solution at room temperature to (S)-(—)-3-ureido-2-methylpropanol-1, characterized as the N,O-diacetate and N,O-di-p-iodobenzoate. Direct hydrogenolysis of 1-dihydrothymidine with sodium borohydride gave N^4-deoxyribosyl-(S)-3-ureido-2-methylpropanol-1, characterized as the tetra-p-nitrobenzoyl and the tetra-p-iodobenzoyl derivatives. A by-product of the hydrogenolysis of 1-dihydrothymidine was isolated as the methyl ether of the carbinolamide. On hydrolysis the racemic 2-oxo-4-methoxy-5-methyl-5,6-hexahydropyrimidine was obtained which on the basis of nmr data was assigned the cis configuration. The photochemical reduction of thymidine, after acid hydrolysis and column chromatography over silica gel, led to 3-ureido-2-methylpropanol-1, characterized as the diacetate, identical with the product having the S configuration obtained via catalytic hydrogenation. A product of the photoreduction is N-deoxyribosylurea which must be formed via photohydration of thymidine (Y. Kondo and B. Witkop).

Photoreduction of Uridine and Reduction of Dihydrouridine with Sodium Borohydride

Irradiation of aqueous solutions of uridine at pH 9.5-10 and 50° with a low-pressure mercury lamp in the presence of a 2.0 M excess of sodium borohydride until 65% or less of the absorbance at 260 mμ had disappeared leads to dihydrouridine, which was crystallized for the first time, and only minor amounts of the products resulting from photohydration and by light-independent hydrogenolysis by excess borohydride. The product of the secondary (dark) reaction, N-(β-D-ribofuranosyl)-N-(γ-hydroxypropyl)urea, was characterized as the penta-p-iodobenzoate, mp 248°. The hydrogenolysis product from dihydrouracil and borohydride, γ-ureidopropanol, mp 62°, was characterized as the N,O-bis-p-nitrobenzoyl derivative and its structure proven by oxidation to γ-ureidopropionic acid, identical with the product of basic ring opening of dihydrouracil. Pyrolysis of γ-ureidopropanol gave the cyclic urethan (P. Cerutti, Y. Kondo, W. Landis, and B. Witkop).

Photolysis of Dihydrothymidine

Irradiation of an aqueous unbuffered solution of 1-dihydrothymidine for 27-30 hr with a high pressure mercury lamp leads to complete disappearance of the UV absorption at 230 mμ. Purification by column chromatography led to isolation of N_1-deoxyribosyl-N_1-n-propylurea, n-propylurea and (S)-dihydrothymine. Dihydrothymine on photolysis gave n-propylurea and urea. Under identical conditions the photolysis of dihydrouridine led only to minor cleavage of the ribosyl residue and to the isolation of 5% of dihydrouracil (Y. Kondo and B. Witkop).

Cyclothym(id)ine

Thymine isomers, containing a three-membered ring, are of considerable interest not only chemically, but also biologically. They were first synthesized by the reaction of 1,3-disubstituted uracils with dimethyloxosulfonium methylide in good yields.

These isomers are very sensitive to UV irradiation and easily converted to seven-membered rings which are also unstable (Y. Kondo and B. Witkop).

Synthetic Polypeptides as Substrates and Inhibitors of Collagen Proline Hydroxylase

The synthetic polypeptide ($[3,4\text{-}^3H\text{-Pro}]\text{-Gly-Pro})_n$ can be hydroxylated by collagen proline hydroxylase. The affinity of the enzyme for the substrate increases with increasing molecular weight of substrate over the range 1300 to 8000. The data indicate that maximal affinity should be observed with a polypeptide of molecular weight considerably greater than 8000. Poly-L-proline II and $(\text{Pro-Gly-Pro})_n$ appear to be competitive inhibitors of the hydroxylation reaction. The series of oligopeptides, $(\text{Gly-Pro-Ala})_n$, $(\text{Gly-Pro-MePro})_n$, $(\text{Pro-Gly-Pro})_n$ and $(\text{Pro-Gly-FPro})_n\text{Pro}$, were tested for inhibition of hydroxylation over the range n=1 to n=4. Inhibition is first observed at n=2 and for these short oligopeptides is maximal between n=3 and n=4. Secondary structure appears to be less important than primary structure in determining whether a peptide will be an inhibitor of the hydroxylase. It is suggested that in a polypeptide chain a specific sequence of 6 to 9 amino acids is required to identify an appropriate prolyl residue for hydroxylation. Among a series of proline derivatives tested, only 4-fluoro, 3- and 4-methylproline inhibited the hydroxylase (J. Hutton, Jr., A. Marglin, B. Witkop, J. Kurtz, A. Berger, and S. Udenfriend).

Nisin

Nisin may be fragmented in two ways by treatment with cyanogen bromide under different conditions. The cleavage of the methionyllysine bond is suppressed at 0°C. The same peptide bond may be cleaved with cyanogen bromide at 37°C. The various fragments of nisin resulting from the different modes of cleavage have been separated by gel filtration and countercurrent distribution. Three of the five residues of lanthionine in nisin remain with the H_2N-terminal fragment, two are found in the COOH-terminal fragment. The H_2N-terminus of the COOH-terminal fragment is lysine. The following partial sequence may be written for nisin: $-HN-\underset{\underset{CHR}{\|}}{C}-(\text{Gly,Lan,Leu})\text{Met-Gly-Asp-Lan-Met-Lys}$ original NH_2-terminus: isoleucine; original COOH-terminal sequence: dehydroalanyllysine.

There are present in nisin three residues of α,β-unsaturated amino acids, two of these are dehydroalanine, one is β-methyldehydroalanine (E. Gross, P. Lee, N. Dalgard, J. Morell, and B. Witkop).

Automation of Amino Acid Analyses

1. Automatic Integration: Two electronic integrators have been used extensively in the calculation of the amino acid content of peptide and protein hydrolysates.

2. Automatic Column Regeneration: One analyzer is fully equipped for automatic column regeneration immediately after completion of an analysis and can prepare the column for essentially continuous operation.

3. Automatic Sample Application: A multiple sample applicator of manual operation is the prototype of the Automatic Sample Applicator. The Multiple Sample Applicator accepts four samples at one time. Semicontinuous operation is possible by merely advancing the next sample into the buffer supply line to the column. The construction of the Automatic Sample Applicator in partial collaboration with the Biomedical Engineering Section of the Division of Research Services is underway and presently being tested. The final version of automatic sample application calls for the use of two columns and alternate sample admission.

4. Automation of ninhydrin analysis of column effluents has been improved. The existing "robot chemist" is to be replaced by a redesigned model (E. Gross and J. Morell).

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

During the past year, 8-3/4 man years were expended in the performance of some 14,000 individual determinations. About 9,000 elemental and functional group analyses were performed using micro, ultramicro and semimicro techniques involving the following types of analyses: gravimetric, volumetric, colorimetric, coulometric, gasometric, and potentiometric. Over 5,000 instrumental analyses were performed by: UV, IR, NMR, ORD, and Mass Spectrometry, Flame Photometry and Vapor Pressure Osmometry. Service is routinely provided to about 150 scientists at NIH and to perhaps 20 investigators in other government agencies which lack adequate facilities to do their own analytical work.

A high resolution Mass Spectrometer (Hitachi) was put into operation in February. Low and high resolution mass spectrometry is now offered as a regular service and in the near future mass spectra of gas chromatography effluents will be available on request.

SECTION ON STEROIDS

Microbial Hydroxylation of (25R)-22,26-imino-5α-cholest-22(N)-en-3β-ol (Solacongestidine)

The alkamine solacongestidine isolated from Solanum congestiflorum was incubated with a number of strains of fungi known for their ability to hydroxylate steroids in the C-16α or β position. No 16-hydroxylated product was isolated. However, a substance recovered from the broth of Corticium centrifugum possessed characteristics of a C-11β hydroxylated product along with dehydrogenation at C-3. Thus, the structure (25R)-22,26-imino-11β-hydroxy-5α-cholest-22(N)-en-3-one is tentatively assigned to the fermentation product. (H. Kataoka and Y. Sato)

The Study of the Alkaloids from Solanum congestiflorum (Natri)

The four alkaloids isolated from Solanum congestiflorum, i.e., (I) (25R)-22,26-imino-5α-cholest-22(N)-en-3β-ol; (II), (25R)-22,26-imino-5α-cholest-22(N)-en-3β,16α-diol; (III and IV), (25R)-22,26-imino-5α-cholest-22(N)-23 and 24-on-3β-ols have been characterized by more confirmatory data. Of particular interest is the isolation of 3β-hydroxybisnorallocholanic acid from the alkaline treatment of compound IV. (H. Kataoka and Y. Sato).

Photosensitized Hydration of Cholesterol and Related Steroid Olefins

Irradiation of cholesterol in t-butyl alcohol solution containing water and o-xylene (sensitizer) gave two compounds in excellent yield. Preliminary data indicate the reaction products are a diol and an oxide. Photodeuteration experiments gave two compounds analogous to the above. Mass spectral data indicate two deuterium atoms are present in each compound. (J. A. Waters and B. Witkop).

Photorearrangement and Sodium Borohydride Reduction of 1,4-cholestadien-3-one

Irradiation of 1,4-cholestadien-3-one (I) in t-butyl alcohol gave 4 phenols, i.e., 1-hydroxy-4-methyl-19-norcholesta-1,3,5(10)-triene (II, 40%), 3-hydroxy-1-methyl-19-norcholesta-1,3,5(10)-triene (III, 40%), 4-hydroxy-2-methyl-19-norcholesta-1,3,5(10)-triene (IV, 10%), and 2-hydroxy-4-methyl-19-norcholesta-1,3,5(10)-triene (V, 4%). Irradiation of I in the presence of sodium borohydride afforded phenols II, III, IV, and 4-methyl-19-norcholesta-1,3,5(10)-triene. Ground state borohydride reduction of I gave 5β-cholest-1-en-3-one (10%), 4-cholesten-3-one (18%), 4-cholesten-3β-ol (40%) and a hydrocarbon. The formation of phenols in lieu of sodium borohydride reduced compounds in this irradiation-borohydride reaction is in direct contrast to the photoreduction of 4-cholesten-3-one (previous study) in which the compounds formed were essentially the same as the ground state reduction products. (J. A. Waters and B. Witkop).

The Degradation and Structural Elucidation of the Triterpenoid, Carpesterol

The lipid fraction of Solanum xanthocarpum provides a crystalline triterpenoid called carpesterol. As a part of the continuing structural elucidation, the free hydroxyl group of carpesterol was oxidized to the ketone which gave a derivative whose RD curve was similar to carpesterol suggesting that the hydroxyl is on the sidechain. The mass spectrum of the ketone corroborated this evidence.

Systematic replacement of the oxygen in carpesterol with hydrogen, excepting the oxygen attached to the 3-position, led to a series of derivatives that were non-identical to the corresponding series of the lanosterol, euphol, tirucallenol, dammaranol, and 4-methylfusidanol groups. Thus, a unique tetracyclic triterpenoid skeleton is manifest. (J. Beisler and Y. Sato).

The von Braun Degradation of Demissidine

The well known steroidal alkaloid, solanidine, and its dihydro derivative, demissidine, have been resistant to degradation in the vicinity of the tertiary nitrogen atom. Thus, earlier workers were unsuccessful in degrading solanidine using the von Braun procedure with cyanogen bromide. In this laboratory it was found that a bromocyanamide could be obtained from demissidine acetate using the von Braun reaction. Reduction of the bromocyanamide with $LiAlH_4$ returned demissidine and also (20S:22R:25S)-22,26-imino-5α-cholestan-3β-ol. The bromocyanamide could be developed into a key synthetic intermediate for conversion into other steroidal alkaloids. (J. Beisler and Y. Sato).

Metabolism of Progesterone-4-C^{14} by Adrenals from Rats with a Mammotropic Pituitary Tumor

Previous studies from this laboratory reported the *in vitro* conversion of progesterone-4-C^{14} by adrenal glands from rats bearing transplantable mammotropic pituitary tumors (MtT). When compared with normal rat adrenal tissue, it was found that a marked decrease in progesterone conversion by adrenal tissue from MtT rats occurred, and isolation of metabolites strongly indicated that different biosynthetic pathways were involved. The normal adrenal gland tissue produced corticosterone (B) and 18-hydroxydeoxycorticosterone (18-OH-DOC) whereas the MtT tissue produced only 18-hydroxycorticosterone (18-OH-B).

The effects of drugs that inhibit 11β-hydroxylation (Metopirone) and conversion of cholesterol to pregnenolone (Eliptin) on the biosynthetic pathways in these stressed adrenals is at present under study. (D. F. Johnson and D. Francois).

Adrenal Metabolism of the Gerbil

Progesterone-4-C^{14} was incubated *in vitro* with gerbil adrenal tissue from animals treated for extended periods with the hypocholesterolemic agent triparanol (MER-29). Other investigators have demonstrated that the principal mode of action of this drug in lowering blood cholesterol is *via* inhibition of conversion of desmosterol to cholesterol. Also it was shown earlier that atherogenic effects in the gerbil were less, and the lowering of blood cholesterol occurred only after prolonged treatment as compared to the effects in rats.

In the present study it was found that triparanol treatment resulted in marked inhibition of 21-hydroxylation of progesterone. Little effect was observed in 11β-hydroxylation. The data emphasize the importance of direct evaluation of the effects of hypocholesterolemic drugs on the adrenal gland as well as for the production of a decreased cholesterol pool. (D. Francois and D. Johnson).

The Synthesis of Solacongestidine

The synthesis of solacongestidine and its analogues, a possible biogenetic precursor of solanidine, solasodine and other related steroidal alkaloids will be attempted by the reaction of 3β-acetoxy-20α-bromo-5α-pregnane with α-methyl-δ-chlorovaleronitrile or 5-methyl-2-piperidone. An attempt to prepare 3β-acetoxy-20α-bromo-5α-pregnane by the treatment of 3β-acetoxy-5α-pregnan-20β-ol with phosphorus pentabromide led to D-homoannulation to afford 3β-acetoxy-17aα- and 17aβ-bromo-17α-methyl-D-homo-5α-androstanes. Chlorination with phosphorus pentachloride, sulfuryl chloride and thionyl chloride also led to D-homoannulation. (R. T. Li and Y. Sato).

Serial No. NIAMD-LC-1
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Oxidation Mechanisms in Metabolic Processes

Previous Serial Number: NIAMD-LC-1

Principal Investigator: Louis A. Cohen

Other Investigators: John W. Thanassi

Cooperating Units: None

Man Years (computed for the 12 month period)

Total: 1
Professional: 1
Other: 0

Project Description:

A study of the mechanisms by which molecular oxygen is enzymatically activated for direct incorporation into substrate; the mechanisms by which the energy derived from oxidative processes is converted into labile bond energy, as in the pyrophosphate linkage.

Objectives: To demonstrate that when oxygen is bound to metal-chelate systems, it is transformed from the triplet (diradical) state to the singlet (ionic) state and that the direct incorporation of oxygen into substrate involves the electrophilic attack of activated singlet oxygen. To study possible mechanisms for the conservation of oxidative energy by means of chemical models and to apply such knowledge to elucidation of the enzymatic pathways.

Methods Employed: Infrared and ultraviolet spectroscopy are used to follow rates of reaction of intermediates and to determine structural characteristics. The latter method is also used for quantitative assay of labile materials. Nuclear magnetic resonance spectroscopy is used for structural elucidation of closely related materials. Various chromatographic procedures, particularly on thin layer plates, are used extensively.

Major Findings: Oxidation of hydroquinone monoacetate in inert solvents, in the presence of halide ion, leads to the formation of acetyl halides in high yield. The reaction shows that a considerable portion of the available oxidative energy may be trapped as bond energy in stable organic compounds of very high free energy content. Furthermore, this finding sheds light on the mechanism of the reaction, indicating it to proceed through an acylium ion pair.

The rate of lactonization of o-coumaric acid has been studied at pH 5. The half-reaction time of 15 minutes demonstrates that hydroquinones can, in principle, esterify spontaneously with protein carboxyl groups. Experimental evidence from model systems is now complete for the proposed scheme of oxidative phosphorylation.

Significance to Bio-medical Research and the Program of the Institute: The mechanism by which molecular oxygen is carried into the cell, bound to hemoglobin, has been well elucidated. Its subsequent fate remains obscure. One immediate goal of this project is to determine the mechanisms by which oxygen is activated to a state in which it can attack a variety of relatively inert substrates.

Similarly, the function of ubiquinone in oxidative phosphorylation has yet to be elucidated. It has been shown that oxidative energy is utilized in the conversion of ADP to ATP in mitochondria, ubiquinone being an essential cofactor.

The present findings permit a novel role to be assigned to ubiquinone, Vitamin K and other quinone cofactors.

Proposed Course of Project: Oxygenated synthetic metal-chelate systems will be prepared and their ability to transfer oxygen to various acceptor molecules will be studied. Studies will be continued on bond conservation of oxidative energy and efforts will be made to link the chemical and mitochondrial processes.

Honors and Awards: None

Publications:

Thanassi, J. W., and Cohen, L. A.: The Conservation of Oxidative Energy. Formation of Acyl Anhydrides *via* the Oxidation of Hydroquinone Monocarboxylic Esters. *J. Am. Chem. Soc.* 89: 5733, Oct. 1967.

Serial No. NIAMD-LC- 2
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemical Modification and Cleavage of Proteins

Previous Serial Number: NIAMD-LC-2

Principal Investigator: Louis A. Cohen

Other Investigators: S. Isoe, and S. Takahashi

Cooperating Units: H. Junek, Guest Worker from the University of Graz, Austria

Man Years (computed for the 12 month period)

Total: 1-1/2
Professional: 1-1/2
Other: 0

Project Description:

A study of the chemical modification and fragmentation of polypeptides and proteins by use of selective chemical and physico-chemical reagents. The elucidation of sequence, tertiary structure and protein topology by such methods.

Objectives: To effect the splitting of complex peptides by chemical or electrochemical oxidation of tyrosine and concomitant labilization of the adjacent peptide bond. To develop conditions which lead to specific and selective attack at the tyrosyl moiety. To demonstrate the utility of the technique in splitting long peptide chains into shorter fragments, facilitating sequence determination. To utilize such oxidative methods for selective modification of function groups on the surface of a globular protein and thus permit the probing of the surface structure. To modify proteins by selective oxidation or reduction techniques.

Methods Employed: Ultraviolet spectroscopy is used to follow the course and extent of cleavage reactions. Infrared spectroscopy is used to elucidate the structure of reaction products. Paper and column chromatography, ion exchange and high-voltage electrophoresis are used to separate and purify polypeptide fragments. An automatic amino acid analyzer is used to follow the extent of chemical reaction and the purification of peptide fragments. Gas chromatography assists in quantitative end-group assay. Various techniques are used for assay of enzyme activity, including spectral and automatic pH devices. Electrochemical oxidation is effected by use of controlled voltage equipment coupled with automatic pH control.

Major Findings: Exposure of N-terminal pyroglutamyl peptides to diborane in tetramethylurea solution leads to partial reduction of the pyrrolidone ring to proline. This conversion of a non-basic residue to the basic proline permits the facile identification of the N-terminal residue as well as an introduction into sequence determination. The method has been demonstrated on the polypeptide hormone, gastrin.

Significance to Bio-medical Research and the Program of the Institute: Despite the remarkable achievements of the past several years in the X-ray crystallography of proteins, there exists no satisfactory method for determining three-dimensional structure in solution, under conditions of enzymatic activity. The use of a chemically reactive polymer or a platinum electrode, capable of selectively and rapidly modifying functional groups only on the surface, may provide a solution to the problem.

Proposed Course of Project: Extension of the continuous flow electrolysis technique to other proteins is planned. The objective is to utilize information obtained by selective oxidation and reduction of proteins to construct three-dimensional models of their conformation in solution.

Honors and Awards: None

Publications:

Cohen, L. A., and Farber, L.: Cleavage of Tyrosyl-Peptide Bonds by Electrolytic Oxidation. Methods in Enzymology 11: 299, 1967.

Isoe, S., and Cohen, L. A.: The Selective Modification and Cleavage of Tyrosyl-Peptide Bonds by Continuous Flow Electrolysis. Arch. Biochem. Biophys., in press.

Cohen, L. A.: Group Specific Reagents in Protein Chemistry. Ann. Rev. Biochem.: Sept. 1968.

Serial No. NIAMD-LC-3
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Cleavage of Peptide Bonds by Intramolecular Participation

Previous Serial Number: NIAMD-LC-3

Principal Investigator: Louis A. Cohen

Other Investigators: Kenneth L. Kirk, and Wolfgang Schreiber

Cooperating Units: None

Man Years (computed for the 12 month period)

Total: 2-1/4
Professional: 2
Other: 1/4

Project Description:

A study involving the synthesis and utilization of various polyfunctional reagents for specific peptide bond hydrolysis.

Objectives: To synthesize reagents which are capable of reacting rapidly and selectively with terminal amino groups and sulfhydryl groups of proteins; to utilize powerful nucleophiles present in the reagents for acid- and base-catalyzed hydrolysis of peptide bonds by intramolecular participation.

Methods Employed: Chromatographic techniques are utilized to separate and purify reagents and products. Nuclear magnetic resonance, infrared, ultraviolet and mass spectroscopy are used to analyze, characterize and assay materials as well as to follow reaction kinetics.

Major Findings: The peptide bond of 1,2-dimethyl-5,7-dinitrobenzimidazolyl-4-alanylglycine has been found to hydrolyze, at 50°, 200,000 times as fast as that of 2,4-dinitrophenylalanylglycine. Even more impressive results have been obtained with 2-amino-4,6-dinitrophenyl peptides: the rate of peptide cleavage, at 25° and pH 7-8 is too fast to measure by normal techniques and probably exceeds the rate of enzyme proteolysis by a large factor. The latter constitutes the most efficient model of enzyme proteolysis discovered to date. Studies of the mechanism by which the intramolecular catalysis occurs suggest that enzymatic hydrolysis of the esters and amides do not necessarily occur by parallel mechanisms and that the two types of substrates may involve entirely different catalytic sites on the enzyme. It is especially noteworthy that bases with pK values as low as 2 or 3 are extremely powerful catalysts in their protonated forms.

The same reagents have been explored for end-group identification and amino acid sequence determination. Although certain obstacles remain to be overcome, the rapidity and technical ease with which degradation may be accomplished make these reagents competitive with those currently in use for analytical purposes.

Activated haloimidazoles, such as 4-bromo-5-nitroimidazole, react specifically with sulfhydryl groups of proteins, sulfur replacing the halogen atom. The resulting colored derivatives of the proteins may be used as "reporters", to measure the polarity and ionic character of the microenvironment and as tracers during the enzymatic degradation of the proteins.

Significance to Bio-medical Research and the Program of the Institute: The only chemical method for peptide sequencing, the Edman procedure, is a multi-step process which has limited utility. Development of a simpler technique, requiring less technical manipulation, effecting quantitative cleavage and amenable to solid state applications, would be extremely useful for sequence determination of peptides and proteins.

Proposed Course of Project: Present indications are that reagents of the type described will be useful for such a purpose. Further exploration is needed to find the most effective reagent for the purpose, i.e., one which can effect peptide bond cleavage under the mildest possible conditions.

Honors and Awards: None

Publications:

Milne, G. W. A., and Cohen, L. A.: Nuclear Magnetic Resonance and Mass Spectra of Cycloserine Derivatives. Tetrahedron 23: 65, 1967.

Serial No. NIAMD-LC-4
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Synthesis of Carbohydrate Derivatives for Medical Research

Previous Serial Number: NIAMD-LC-4

Principal Investigator: H. G. Fletcher, Jr.

Other Investigators: H. W. Diehl, T. Ishikawa (to 11/14/67), D. E. Kiely, T. Kuzuhara (to 8/21/67), R. K. Ness, W. L. Salo (from 9/5/67), E. Zissis

Cooperating Units: None

Man Years

Total: 7 1/4
Professional: 5 1/4
Others: 2

Part A. Steric Control in the Synthesis of Nucleosides

Project Description:

Objective: The demand by many and varied research programs for synthetic nucleosides called for a reexamination of the methods whereby nucleosides of specific anomeric configuration may be synthesized.

Major Findings: In view of the known mechanism of participation of acyloxy groups in nucleophilic displacements on adjacent carbon atoms it is widely assumed that condensation of acylated glycofuranosyl halides with nitrogeneous bases gives nucleosides in which the aglycon is situated *trans* to the substituent at carbon 2. Actually, inspection of the literature shows that, while such *trans* nucleosides normally preponderate in these syntheses, traces of the corresponding *cis* nucleosides have been isolated. A very careful examination of a typical synthesis has now shown that the proportion of *cis* nucleoside may actually be substantial: the condensation of 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl bromide with 5,6-dimethylbenzimidazole gives the *trans* nucleoside derivative 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-5,6-dimethylbenzimidazole in 51% yield and the *cis* nucleoside derivative 1-(2,3,5-tri-*O*-benzoyl-α-D-ribofuranosyl)-5,6-dimethylbenzimidazole in 28% yield.

In earlier reports, the deliberate synthesis of the cytotoxic *cis* nucleoside, 9-β-D-arabinofuranosyl-adenine, through the condensation of 2,3,5-tri-

O-benzyl-α-D-arabinofuranosyl chloride with N-benzoyladenine was described. That this technique may be of general value has now been shown through the syntheses of 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole, a hydrolytic fragment from vitamin B_{12}. Condensation of 2,3,5-tri-O-benzyl-D-ribofuranosyl chloride with 5,6-dimethylbenzimidazole gave 1-(2,3,5-tri-O-benzyl-α-D-ribofuranosyl)-5,6-dimethylbenzimidazole in 66% yield; removal of the masking groups from this nucleoside was virtually quantitative.

In the course of the above research, an improved method for the synthesis of highly reactive glycosyl halides was developed. Such halides are normally prepared through the action of hydrogen chloride or hydrogen bromide on a sugar ester, an acyloxy group being replaced by a halogen atom. The carboxylic acid thus liberated interferes with the subsequent use of the glycosyl halide for nucleoside synthesis and must be removed. Its removal, however, is rendered difficult if the glycosyl halide is neither crystalline nor stable. Work outside NIH (by Dr. W. Zorbach) showed that the p-nitrobenzoates of the sugars are particularly well suited for the preparation of glycosyl halides since treatment of these in dichloromethane solution with hydrogen chloride or hydrogen bromide caused precipitation of the relatively insoluble p-nitrobenzoic acid which could be removed by filtration, leaving a solution of the desired glycosyl halide. It has now been found that p-phenylazobenzoic acid is even less soluble in dichloromethane than is p-nitrobenzoic acid and the use of p-phenylazobenzoates of the sugars for the preparation of glycosyl halides offers some interesting advantages as will be seen in the next following research project.

Significance to Biomedical Research and the Program of the Institute: A broader understanding of the advantages of and pit falls in the traditional method for the synthesis of trans nucleosides has been gained. A method for the synthesis of cis nucleosides has been extended to the synthesis of the only known α-ribonucleoside and should make this material, a component of vitamin B_{12}, readily available for biomedical research.

Part B. The Synthesis of Specifically Blocked Ribonucleosides

Project Description:

Objective: The central role of nucleosides in a variety of biochemical situations has led to the need for nucleosides which are selectively masked at positions in the sugar moiety. In the past, most of such masking has been carried out on the intact nucleoside. The purpose of the present research was to explore the feasability of introducing specific masking in the sugar moiety prior to the establishment of the glycosidic bond. An allied objective was the exploration of a method for separating and purifying the products arising in nucleoside synthesis.

Major Findings: Crystalline 3,5-di-O-benzyl-β-D-ribofuranose was synthesized from D-ribose; its 1,2-di-O-p-phenylazobenzoyl derivative was treated with hydrogen chloride in dichloromethane solution to give 3,5-di-O-benzyl-2-O-p-phenylazobenzoyl-D-ribofuranosyl chloride which was condensed with 5,6-

dimethylbenzimidazole. The mixture of colored products which was formed was readily resolved by chromatography to yield 1-(3,5-di-O-benzyl-2-O-p-phenylazobenzoyl-β-D-ribofuranosyl)-5,6-dimethylbenzimidazole in 61% yield and its α anomer in 10% yield. Removal of the acyl group from these derivatives yielded D-ribonucleosides which are masked at C-3 and C-5 of the sugar moiety. It should be pointed out that the p-phenylazobenzoyl group in this synthetic scheme performs a number of functions. The insolubility of the p-phenylazobenzoic acid in dichloromethane makes the preparation of the glycosyl halide simple and efficient; the p-phenylazobenzoyl group at C-2 in the glycosyl halide assures that the trans nucleoside is the major product of the condensation and, finally the color which this group confers makes chromatographic separation of the anomeric nucleosides (and other products of the condensation) rapid and convenient. A similar synthesis using the benzoyl group in place of the p-phenylazobenzoyl group proved less efficient but led to nucleosides masked with a benzoyl group at C-2 in the sugar moiety. The use of 3,5-di-O-benzyl-D-ribofuranosyl chloride was also studied.

Significance to Biomedical Research and the Program of the Institute: The availability of a method for the synthesis of partially blocked nucleosides should be of assistance in the synthesis of oligoribonucleotides.

Part C. Selective Cleavage of the Glycosidic Bond in Acetylated 2-Acetamido-2-deoxy-β-D-glucopyranosides by a Chemical Transglycosylation

Project Description:

Objective: In earlier work (see 1966-1967 report), it was shown that acetylation of a 2-acylamino-2-deoxyaldose with acetic anhydride and in the presence of anhydrous zinc chloride led to the formation of an acetylated oxazoline. This observation suggested that derivatives of 2-acylamino-2-deoxyaldoses, with a trans arrangement of the groups at C-1 - C-2, may be more susceptible than other aldose derivatives to the action of certain cleaving reagents. The objective of this project was to explore this point.

Major Findings: Preliminary studies clearly showed that acetylated oxazolines related to the 2-acylamino-2-deoxyaldoses are inconveniently reactive for the purposes in mind. Since they react readily with alcohols to give relatively stable glycosides, the behavior of a number of acetylated aldose derivatives with zinc chloride and benzyl alcohol was investigated. At 125° in butyl acetate solution 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-β-D-glucopyranose is rapidly converted into benzyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside in high yield; its anomer reacts much more slowly. The trans glycoside methyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside undergo a similar transglycosylation while its cis anomer is unattacked; methyl β-D-glucopyranoside is similarly stable under these conditions. The intersaccharidic linkage in chitobiose octaacetate is cleaved although a major product of the treatment is a mixture of the acetylated, anomeric benzyl glycosides derived from chitobiose.

Significance to Biomedical Research and the Program of the Institute: The procedure developed should be useful for the selective cleavage of oligosaccharides containing trans 2-acylamino-2-deoxyaldose moieties. It should also be of value in the synthesis of trans glycosides of the 2-acylamino-2-deoxyaldoses.

Part D. Synthesis of D-xylo-Hexos-5-ulose 6-Phosphate

Project Description:

Objective: D-Glucose 6-phosphate is incorporated without fragmentation in the synthesis of myo-inositol by several biological systems and D-myo-inositol 1-phosphate has been shown to be an intermediate in one system and another system has been shown to be NAD-dependent. In view of these facts, it has been suggested that D-xylo-hexos-5-ulose 6-phosphate (5-ketoglucose 6-phosphate) is the key intermediate. The objective of this research was the chemical synthesis of this substance for biochemical testing.

Major Findings: The known 3-O-benzyl-1,2-O-isopropylidene-6-O-triphenylmethyl-α-D-glucofuranose was oxidized with dimethyl sulfoxide to give 3-O-benzyl-1,2-O-isopropylidene-6-O-triphenylmethyl-α-D-xylo-hexofuranos-5-ulose which was isolated in 91% yield. The triphenylmethyl group was removed by acidic hydrolysis and the 3-O-benzyl-1,2-O-isopropylidene-α-D-xylo-hexofuranos-5-ulose thus formed was converted into its dimethyl acetal. After phosphorylation at C-6 with diphenylphosphochloridate, the benzyl and phenyl groups were removed by hydrogenolysis and the resulting 1,2-O-isopropylidene-α-D-hexofuranos-5-ulose dimethyl acetal 6-phosphate was isolated as its di(cyclohexylammonium) salt. Mild acidic hydrolysis of this salt afforded D-xylo-hexos-5-ulose 6-phosphate whose identity was confirmed in a variety of ways. Specimens have been supplied to Dr. F. Eisenberg, Jr. (LBM) for biochemical studies.

Significance to Biomedical Research and the Program of the Institute: Considering the ubiquity of myo-inositol in living tissues, any step which may throw light on the biosynthesis of this substance is significant.

Part E. Syntheses with Partially Benzylated Sugars

Project Description:

Objective: To explore the synthetic potentialities of sugars which are benzylated save at the reducing carbon and at the carbon bearing the lactol ring.

Major Findings: Partially benzylated sugars of the special type under investigation have been shown to be useful precursors in the synthesis of ketoses. Thus, reduction of 2,3,5-tri-O-benzyl-D-arabinofuranose (now commercially available) gave 2,3,5-tri-O-benzyl-D-arabinitol in which the primary hydroxyl group was subsequently masked with a trityl group. Oxidation of the secondary hydroxyl and removal of the masking groups then gave

D-*threo*-pentulose (D-xylulose). A similar sequence converted 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose to L-sorbose.

In closely related research, a partially benzylated sugar was found of value in the synthesis of an unsaturated sugar. Treatment of 2,3,4,6-tetra-*O*-benzyl-*N*,*N*-dimethyl-5-*O*-methylsulfonyl-D-gluconamide with sodium acetate in dimethylformamide gave 2,4,6-tri-*O*-benzyl-3-deoxy-L-*threo*-hex-2-enono-1,5-lactone; through a two-step process this substance was reduced to the sugar derivative 2,4,6-tri-*O*-benzyl-3-deoxy-L-*threo*-hex-2-enopyranose.

Significance to Biomedical Research and the Program of the Institute: The new method for the synthesis of ketoses is of general applicability and will make accessible new and, particularly, labeled ketoses. The new route to unsaturated sugars remains a chemical curiosity for the moment.

Part F. The Chemistry of Ketoses

Project Description:

Objective: Although the ketoses are of great biochemical importance, our knowledge of the chemistry of these substances is much scantier than that of the aldoses. In Part E we have already described a new method for the synthesis of ketoses. The present project is designed to extend our general knowledge of the chemistry of the ketoses. Although glycals derived from aldoses have been known for over forty years and have proved to be among the most useful sugar derivatives, no analogous derivative of a ketose had been made. To synthesize and study the properties of such a substance was the main objective of this project.

Major Findings: The known 3-*O*-methylsulfonyl-D-fructose was benzoylated to give two tetrabenzoates. One of these proved to have the desired pyranose structure and was converted into 1,4,5-tri-*O*-benzoyl-3-*O*-methylsulfonyl-β-D-fructopyranosyl bromide. Reaction of this halide with sodium iodide in acetone solution yielded 1,4,5-tri-*O*-benzyl-2,3-dideoxy-D-*erythro*-hex-2-enulopyranose, the first ketose-related glycal having an endocyclic double bond.

Significance to Biomedical Research and the Program of the Institute: A beginning has been made in expanding our knowledge of the ketoses.

Proposed Course of Project: Efforts to contribute to biomedical research through chemical studies of the carbohydrates are being continued.

Honors and Awards: None

Publications:

Kuzuhara, H., and Fletcher, H. G., Jr.: Syntheses with Partially Benzylated Sugars. VIII. Substitution at C-5 in an Aldose. The Synthesis of 5-*O*-Methyl-D-glucofuranose Derivatives. *J. Org. Chem.* 32: 2531-2534 (1967).

Kuzuhara, H., and Fletcher, H. G., Jr.: Syntheses with Partially Benzylated Sugars. IX. Synthesis of a 5-Hexulosonic Acid (5-Ketohexonic Acid) Derivative and Inversion of Configuration at C-5 in an Aldose. J. Org. Chem. 32: 2535-2537 (1967).

Pravdić, N., and Fletcher, H. G., Jr.: The Behavior of 2-Acetamido-2-deoxy-D-galactose with Isopropenyl Acetate in the Presence of p-Toluenesulfonic Acid. Formation of An Unsaturated Aminosugar Derivative. Croat. Chem. Acta. 39: 71-80 (1967).

Rabinsohn, Y., and Fletcher, H. G., Jr.: Syntheses with Partially Benzylated Sugars. X. A New Method for the Synthesis of Ketoses. J. Org. Chem. 32: 3452-3457 (1967).

Ness, R. K., and Fletcher, H. G., Jr.: Syntheses of 1,4,5-Tri-O-benzoyl-2,3-dideoxy-D-erythro-hex-2-enulopyranose, Derivative of a Ketose-Related Glycal Having an Endocyclic Double Bond. J. Org. Chem. 33: 181-184 (1968).

Stevens, J. D., Ness, R. K., and Fletcher, H. G., Jr.: Syntheses with Partially Benzylated Sugars. XI. Studies on the Synthesis of the Anomeric 5,6-Dimethyl-1-D-ribofuranosylbenzimidazoles (Ribazoles). Comparison of the Condensation of 2,3,5-Tri-O-benzoyl-D-ribofuranosyl Bromide and 2,3,5-Tri-O-benzyl-D-ribofuranosyl Chloride with 5,6-Dimethylbenzimidazole. J. Org. Chem. 33: 0000, May 1968 (in press).

Haga, M., Ness, R. K., and Fletcher, H. G., Jr.: Syntheses with Partially Benzylated Sugars. XII. 3,5-Di-O-benzyl-β-D-ribofuranose and its Use as an Intermediate in the Synthesis of Partially Substituted Ribonucleosides. J. Org. Chem. 33: 0000, May 1968 (in press).

Stevens, J. D., and Fletcher, H. G., Jr.: The Proton Magnetic Resonance Spectra of Pentofuranose Derivatives. J. Org. Chem. 33: 0000, May 1968 (in press).

Kuzuhara, H., and Fletcher, H. G., Jr.: Syntheses with Partially Benzylated Sugars. XIII. Unsaturated Derivatives from 2,3,4,6-Tetra-O-benzyl-N,N-dimethyl-5-O-methylsulfonyl-D-gluconamide by a Novel Type of Elimination Reaction. J. Org. Chem. 33: 0000, May 1968 (in press).

Fletcher, H. G., Jr.: Steric Control in the Synthesis of Nucleosides. Trans, N. Y. Acad. Sci., March 1968 (in press).

Serial No. NIAMD-LC-5
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunochemistry of Carbohydrates

Previous Serial Number: None

Principal Investigator: C. P. J. Gluademans

Other Investigators: N. Roy

Cooperating Units: None

Man Years

Total: 2
Professional: 2
Others: None

Subtitle: Investigation of four types of D. pneumoniae, related by an apparent mutation.

Project Description:

Objective: D. pneumococcus type 61 cross reacts with serum to type 31, and will mutate to type M61 in its presence. Type M61 then cross reacts with Type 13, and M61 will revert to the parent 61 if subcultured serially in the presence of serum to type 13 (Laros 1949). We are investigating this relationship, and first of all are elucidating the structures of the type specific capsular polysaccharides occurring on the organisms which are involved.

Methods Employed: All modern and classical techniques are called upon in the elucidation of the structure of these antigens. These involve hydrolysis studies, methylation, glc, mass spectrometry, nmr and synthesis.

Major Findings: A new facile method to prepare pure antigen from D. pneumoniae had been reported previously (C. P. J. Glaudemans and H. P. Treffers, Carbohyd. Res. 4, 181 (1967)) and we have now shown by sedimentation and diffusion measurements (collaboration with Dr. W. R. Carroll, NIAMD) that the antigen from type 31 thus prepared has a mol. wt. of 110,000 and is therefore probably not degraded, as this order of magnitude has been found for the antigens from D. pneumoniae types III and

VIII, as isolated by other (more laborious) means. Hydrolysis of the antigen from type 31 (S-31) yields an aldobiouronic acid, and galactose and rhamnose. The acidic disaccharide was isolated by chromatography. Diborane reduction of the acetate followed by hydrolysis gave glucose and galactose. Sodium borohydride reduction of the diborane reduced material, followed by hydrolysis yielded glucose and galactitol. From the known stability of urononyl-hexose disaccharides, therefore, the compound must be glucuronosyl-galactose.

Diborane reduction of the polysaccharide (after acetylation) gave a completely neutral polymer, which upon reduction yielded glucose, rhamnose and galactose in the ratio of 1:2:2. The polysaccharide hydrolyzes very easily, making it probably that at least one hexose moiety occurs in the furanoside form. The sedimentation and ultracentrifuge behavior referred to above (mol. wt. studies) show that the material is substantially linear, that is to say, not heavily branched. Also, the antigen shows a behavior compatible with a gaussian molecular weight distribution, as is expected for a polysaccharide.

Methylation studies - to deduce the position of the linkages - are in progress.

Subtitle: Fine structure and immuno dominance in antigenic polysaccharides

Objective: The capsular polysaccharide of D. pneumoniae type 34 (S-34) contains a number of different groupings (galactofuranose, pyranose, glucose, ribitol, phosphate and 0-acetyl groups). The structure is nearly fully known (Baddiley, Buchanan et al), including the sequence of the various sugars. It is therefore excellently suited to study immunodominance of various haptenic groups in the chain.

Major Findings: Two structural features of S-34 are still not clear. The linkage positions between the rabitol and the phosphate, and the position of the 0-acetyl group on the internal galactofuranose residue. We synthesized methyl 6-0-acetyl galactofuranoside for the first time (the structure was rigorously proved) and were able to show that this compound was a good inhibitor of the homologous S-34/rabbit anti 34 precipitin reaction, whereas the parent 6-OH compound was not. This almost certainly shows that the acetate group in the antigen is in the 6-position. Also we prepared (the methyl α-glycoside of) 3-0-β-D-galactofuranosyl-D-glucose, a moiety of S-34. Results indicate that it is not immunodominant, an expected result, since in S-34 galactofuranose carries the phosphate group at 0-3, and that is almost certain to influence the immunochemistry strongly.

Also, the reducing disaccharide was synthesized, but its structure could not be proved beyond a shadow of a doubt. It was found by us that it is possible to have acetyl migration during the Kochetkov condensation of 3,5,6-tri-0-acetyl-1,2-0-methylorthoacetyl-α-D-galactofuranoside with 1,2,4,6-tetra-0-acetyl glucose. Consequently we searched for a glucose derivative not showing any acetyl migration between 3,4. Benzyl-4,6-benzylidene-2-0-acetyl-β-D-glucopyranoside is such a compound.

Subtitle: The synthesis of various aldobiouronic acids possibly occurring in the antigen from D. pneumoniae Type II.

Objective: The structure of the capsular polysaccharide from D. pneumonia Type II has recently been elucidated. Several linkages are still uncertain. It has long been known that S-II cross reacts heavily with dextrans, possibly because the internal glucuronic acid 1 → 6 glucose has an α-linkage. We wished to verify this by inhibition studies.

Major Findings: We Synthesized 6-0-α-D-glucuronosyl-D-glucose (I) (hitherto unknown) as well as the β analogue (II) and the α 1 → 4 analogue (III). Synthesis of (I) was initiated from isomaltose followed by protection of the free C'6 hydroxyl and oxidation after removal of the protecting group. The synthesis of II took an identical course, whereas the synthesis of III took place by using 1-6 anhydro maltose which was oxidized at the C'6 hydroxyl following blocking of the other hydroxyls by benzoyl groups. Inhibition studies on the S-II/horse anti S-II system are now in progress (in collaboration with Dr. M. Heidelberger at NYU).

Significance to Biomedical Research and the Program of the Institute: Work on the immuno determinant groupings in biopolymers will contribute to our general knowledge of immunity. The study of four related antigens appears to be a good vehicle to clarify the reasons for different immunochemical behavior due to minor variations in structure. Secondly, once all antigen structures of this system are known, it will be possible to study the changes in metabolic pathways which find their expression in the synthesis of these different antigens.

Proposed Course of Project: The investigation of the fine structure of polysaccharides derived from D. pneumoniae will be pursued with special attention to the problems of the identification of immunochemically significant groupings.

Honors and Awards: None

Publications:

Roy, N., and Glaudemans, C. P. J.: The Synthesis of Three Fully Acetylated Aldobiouronic Acid Methyl Esters, Including 6-*0*-(Methyl 2,3,4-tri-*0*-acetyl-α-D-glucopyranosyluronate)-tetra-*0*-acetyl-β-D-glucopyranose. *J. Org. Chem.* 33: 1559-1562 (1968).

Glaudemans, C. P. J., and Fletcher, H. G., Jr.: Substitution at the 2- or 3-Hydroxyl Group of D-Glucose. *Carbohyd.* Res. 6: 000 (1968) in press.

Serial No. NIAMD-LC-6
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Higher-Carbon Sugars, Anhydro Sugars, Sugar Alcohols, Disaccharides, and Their Derivatives

Previous Serial Number: NIAMD-LC-5

Principal Investigator: N. K. Richtmyer

Other Investigators: I. Johansson (V.S. from 2/14/68)

Cooperating Units: None

Man Years

Total: 2 1/4
Professional: 1 1/4
Others: 1

Project Description:

Objective: To evolve generalizations relating to the physical and chemical properties of the groups of substances named in the project title to their configurations and conformations.

Methods Employed and Major Findings: Studies on naturally occurring higher-carbon sugars were continued, and the purification and identification of an octulose and a nonulose from Pichi tops (the foliage of *Fabiana imbricata*) is currently nearing completion. Further work on akee fruit (*Blighia sapida*) revealed nothing of special interest. A Dowex 50W-X8 (BA^{++}) column fractionation of the polyhydric alcohols from *Sedum spectabile* failed to show any evidence for the presence of D-*erythro*-D-*galacto*-octitol; this octitol has been reported previously as occurring in the avocado.

The alkaline rearrangement of primeverose (6-*O*-β-D-xylopyranosyl-D-glucose), followed by recovery of unchanged primeverose by crystallization and separation of the remaining material on a cellulose column, has yielded syrupy primeverulose (6-*O*-β-D-xylopyranosyl-D-fructose) with $[\alpha]^{20}_{D}$ -27° in methanol. Studies are now in progress to see whether there is any evidence for the presence of primeverulose in extracts of plants that are known to contain primeverose.

Significance to Biomedical Research and the Program of the Institute: As mentioned before, sedoheptulose (D-*altro*-heptulose) and D-*manno*-heptulose (both of which occur in various families of plants) have been found to be of significance in metabolism (both animal and plant) and in studies on diabetes, respectively. The significance of the presence of the other higher-carbon sugars in plants is still unknown.

Proposed Course of Project: We propose to continue the researches on the isolation and identification of the higher-carbon sugars and sugar alcohols from plant sources, as well as on the synthesis and reactions of these and other rare sugars and their derivatives.

Publications: None

Serial No. NIAMD-LC-7
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Synthesis of 6,7-Benzomorphan and 2′-Hydroxy-2-Methyl-6,7-benzomorphan.

Previous Serial Number:

Principal Investigators: Ken Kanematsu, Mikio Takeda, Arthur E. Jacobson, and Everette L. May.

Other Investigators: None

Cooperating Units: Analgesics Testing Laboratory of this Section.

Man Years:

Total: 1 1/4
Professional: 1 1/4
Other: 0

Project Description:

Objectives: The synthesis and pharmacologic evaluation of the parent 6,7-benzomorphan and its 2′-hydroxy-2-methyl relative.

Methods Employed: Usual methods of organic synthesis and more sophisticated techniques recently developed.

Major Findings: Catalytic reduction of ethyl 3-amino-1-naphthalene acetate under a variety of conditions gave saturation of the 5,6,7,8- rather than the 1,2,3,4-positions, so that this route to 6,7-benzomorphan (I) failed. At length a 10-step sequence (from 4-phenylpyridine) was elaborated which gave 2-methyl-6,7-benzomorphan (II) in 5% overall yield. Adverse stereochemistry (1,3-diequatorial, chair conformation) had to be overcome in the cyclization of 2-carboxy-1-methyl-4-phenylpiperidine, the ester of which could not be cyclized presumably because steric inhibitions could not be overcome with the latter. N-demethylation of II to I was effected in 25% yield only by the von Braun cyanogen bromide method or with diethyl azodicarboxylate (35%). Compound II was also converted to the 2'-hydroxy relative (in insufficient quantity for testing) by nitration, hydrogenation and nitrous acid oxidation. The nitration step gave isomeric mixtures. Compound II was also finally obtained (after many trials dating from 1960) in low yield from pyridine by the usual modification of the Grewe morphinan synthesis. Thus contrary to our previous view, it is possible to cyclize a 1-methyl-2-benzyl-1,2,5,6-tetrahydropyridine to a 6,7-benzomorphan when there is not an electron-donating group in the 4-position of the pyridine nucleus, although an alkyl group in this position greatly facilitates polarization of the Δ^3 double bond and thus the generation of a carbonium ion at position 4 and subsequent cyclization.

Compound II, without a quarternary carbon, has codeine-like analgesic activity (comparable to the 5-methyl homolog) and even I has some activity although it is highly toxic.

Significance: The above findings should enable one to assess the role and importance of the quaternary carbon in analgesia and drug dependence of the morphine type. It should open new possibilities for the development of superior pain-relieving agents.

Proposed Course of Project: Improvements in the synthesis of 2-methyl-6,7-benzomorphan and the 2'-hydroxy analog will be sought. Derivatives of these two compounds are contemplated.

Serial No. NIAMD-LC-8
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Aldolase Inhibitors

Previous Serial Number: Same

Principal Investigator: James G. Murphy

Other Investigators: None

Cooperating Units: None

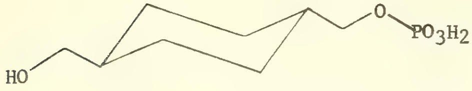
Man Years (computed for the 12 month period):

Total: 1
Professional: 1
Others: 0

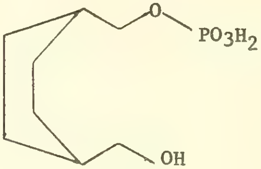
Project Description:

Objectives: The following studies involve potential inhibitors of the glycolytic enzymes for biological study and medical use.

Methods Employed and Major Findings: The trichloroacetontrite method of phosphorylation failed when applied to *cis* or *trans*-1,4-cyclohexanedimethanol. Experimental conditions for direct esterification were than developed leading to a 34% yield of *trans*-1,4-cyclohexanedimethanol monophosphate, I, and a 24% yield of *cis*-1,4-cyclohexanedimethanol monophosphate, II.

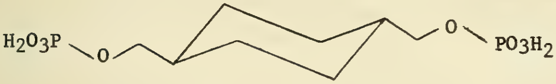


I

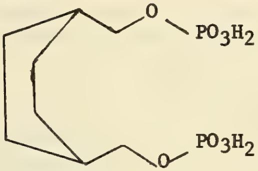


II

Further phosphorylation to *trans*-1,4-cyclohexanedimethanol diphosphate, III, and *cis*-1,4-cyclohexanedimethanol, IV, is limited by the thermal degradation



III



IV

of the substrates. In order to improve yields, infrared radiation was used with a view to increasing the population of transition state intermediates. In one pair of experiments, the photochemical conversion at one hour was four times that of the thermal. In another, a photochemical conversion of 51% was obtained versus 34% for the thermal.

Significance: Since it has been shown (Hartman and Barker, 1965) that the binding energy of hexose phosphates to aldolase involves primarily the anionic centers of the substrates, the above substances should compete with hexose phosphates for this and possibly other enzymes. The monoesters are available for biological evaluation now; the diesters should be available shortly.

Proposed Course of Project: Exploration of alternate routes of phosphorylation and synthesis of analogs of substrates for carbohydrate metabolism.

Publications: None.

Serial No. NIAMD-LC-9
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Titles:
1) The synthesis of 2,3,4,5,6,6a-hexahydro-8-methoxy-3,11-dimethyl-1*H*-4,11b-methanobenzofuro[3,2-*d*]azocine.
2) Transformations in the morphine series. Conversion of thebaine to 1-methyl-3a-(3′-hydroxy-6′-methylphenyl)-4,2′-oxidooctahydroindole by two different routes. A rearrangement *via* an aziridinium intermediate.
3) The synthesis of 2,5,9-trimethyl-azabicyclo[3.3.1]non-6-ene.
4) The synthesis of a N-position isomer of the analgesically active 6,7-benzomorphans.
5) The synthesis of 1,5-methano-2-benzazepine.

Previous Serial Number: Same

Principal Investigators: Michael Mokotoff

Other Investigators: Lewis J. Sargent

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total: 1 1/2
Professional: 1 1/2
Others: 0

Project Description:

Objectives: 1) Although the title compound was reported to have been prepared as described in the previous annual report, further investigations indicated that a molecular rearrangement had taken place. In order to examine the analgesic properties of the title compound, an alternate synthetic approach was utilized which led to the correct structure.

2) To investigate the rearrangement described above that led to an octahydroindole structure, the synthesis of this substance by an independent route, and its identification with degradation product obtained from the alkaloid galanthamine.

3) To synthesize a small partial structure of morphine and determine whether it has any analgesic activity.

4 and 5) To obtain new ring systems that might be useful as analgetics.

Methods employed and Major findings: 1) Reduction ($NaBH_4$)of the keto compound, 2,3,4,5,6,6a-hexahydro-8-methoxy-3,11-dimethyl-1H-4,11b-methanobenzofuro[3,2-d]azocin-12-one, gave the corresponding α-alcohol. The alcohol was mesylated and the mesylate hydrogenolyzed with $LiAlH_4$. This led to an octahydroindole rearrangement product instead of the title compound. The title compound was obtained by a modified Wolff-Kishner reduction (corresponding hydrazone and potassium-t-butoxide). It had an analgesic potency, in mice, of approximately one-half that of codeine.

2) The aforementioned octahydroindole was independently synthesized by starting with 9b-[2-(dimethylamino)ethyl]-3,4,4a,9b-tetrahydro-6-methoxy-9-methyl-1(2H)-dibenzofuranone, a ketone obtained by degradation of thebaine. This ketone on reduction ($NaBH_4$) gave two isomeric alcohols, α and β, which were separated by column chromatography. The stereochemistry of these two alcohols was proved by nmr analysis of their acetates. The mesylate of the α-alcohol, in benzene, gave 1,1-dimethyl-3a-(3′-methoxy-6′-methylphenyl)-4,2′-oxidooctahydroindole, methanesulfonate salt. Ionic exchange gave the bromide salt, which on dry distillation lost methyl bromide and gave the same octahydroindole as described in part 1. This compound is optically active and was correlated with a racemic sample of the same compound as reported by Japanese workers. The analgesic activity in mice of the methoxyoctahydroindole and its phenolic counterpart (obtained by cleavage of the methyl ether with 48% HBr) was measured, the former being inactive and the latter having approximately one-third the activity of codeine.

3) 3,4-Lutidine was converted to its methiodide, which was utilized in two ways: a) reacted directly with allyl magnesium bromide and the intermediate dihydro form reduced to the tetrahydro form with $NaBH_4$, and b) reduced ($NaBH_4$) to the tetrahydro form which was reacted with allyl bromide. The intermediate quarternary salt was rearranged with phenyl lithium. In both cases extensive purification is necessary to obtain 2-allyl-1,3,4-trimethyl-1,2,5,6-tetrahydropyridine.

4) Condensation of anisaldehyde with diethyl succinate in the presence of sodium methoxide gave p-methoxy phenyl itaconic acid. Hydrogenation of the double bond gave p-methoxybenzyl succinic acid. Cyclization of this compound would give the starting intermediate compound, 3-carboxy-7-methoxytetralone-1.

5) Condensation of anisaldehyde with diethylmalonate in the presence of piperidine gave diethyl p-methoxybenzlidene malonate, which on reaction with KCN followed by hydrolysis would give p-methoxyphenyl succinic acid. Cyclization gives the starting intermediate, 3-carboxy-6-methoxy-1-indanone.

Significance: 1) To further the understanding of structural requirements and functional groups necessary for analgesic activity in morphine analogs.

2) The results of the analgesic testing substantiates the theory that for design of active morphine analogs the N-containing ring should be 6-membered. For example, the methoxy-octahydroindole (5-membered N-ring) was inactive as an analgesic in mice while the methoxy-methanobenzofuro[3,2-*d*]-azocine had a potency one-half that of codeine.

3) To determine if a double bond can substitute for the electron-rich aromatic ring in the analgesically active benzomorphan series.

4 and 5) To observe whether or not these novel ring structures will be biologically active.

Proposed Course of Projects: 1 and 2) Completed.

3) To isolate the allyl derivative in a pure form and try various methods of cyclization that would give the azabicyclo[3.3.1]nonene system.

4 and 5) By appropriate elaboration of the intermediates, including introduction of nitrogen into the ring, the desired ring systems could be obtained.

Publications: Mokotoff, M. and Sargent, L. J.: Transformations in the Morphine Series, III. Transformation of Thebaine to Methanobenzofuro[3,2-*d*]azocines. *J. Org. Chem.* In press 1968.

Mokotoff, M.: Transformations in the Morphine Series. IV. Conversion of Thebaine to 1-methyl-3a-(3′-hydroxy-6′-methylphenyl)-4,2′-oxidooctahydroindole by two different routes. A rearrangement *via* an aziridinium intermediate. *J. Org. Chem.* In press 1968.

Sargent, Lewis J. and Joshi, Bhuwan: Transformations in the Morphine Series II. A New Position Isomer of Dihydromorphinone *J. Med. Chem.* 11: 336 (1968).

Serial No. NIAMD-LC-10
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1) Molecular modification of (±)-2′-hydroxy-2,5-dimethyl-6,7-benzomorphan.

2) Synthesis of (±)-2′-hydroxy-2,9-dimethyl-6,7-benzomorphan.

3) Synthesis of D-Glucuronic acid derivatives.

Previous Serial Number: Same

Principal Investigator: J. Harrison Ager

Other Investigators: None

Cooperating Units: Pharmacology Unit of this section

Man Years (computed for the 12 month period)

Total: 3/4
Professional: 3/4
Other: 0

Project Description:

Objectives: 1) Synthesis of N- and O- substituted derivvatives of 6,7-benzomorphan for biological assay.

2) To prepare a new 6,7-benzomorphan without a substituent in the 5-position for evaluation as an analgesic.

3) To synthesize D-Glucuronic acid derivatives in order to effect a reaction with previously prepared 6,7-benzomorphans.

Methods Employed: Standard methods of organic, physical-organic, and analytical chemistry.

Major Findings: 1) The N-benzyl derivative of 2′-hydroxy-2,5-dimethyl-6,7-benzomorphan was prepared by the Grewe morphinan synthesis after repeated failure of the more versatile alternative synthesis based on the Stevens rearrangement of 1-benzyl-1,2,5,6-tetrahydropyridines. Thus 4-methyl-pyridine benzylbromide and p-methoxybenzylmagnesium chloride were brought together in the Freund reaction to give a dihydropyridine derivative which was reduced with sodium borohydride in base giving the tetrahydro compound. Treatment with hot 48% HBr and 30-33% HBr-HAc gave the desired N-benzyl compound in low yield.

The N-H derivative (nor compound) was prepared from the N-benzyl derivative by catalytic hydrogenation over PtO_2 in methanol. The usual method for removal of one alkyl group from tertiary amines (v. Braun) cyanogen bromide as the reagent, failed to yield the desired nor compound. The screening of these derivatives is in progress with preliminary reports showing that the nor compound (N-H) has no analyesic activity at sub-toxic doses. The methyl ether of the N-methyl analog showed good activity, subcutaneously.

2) Attempts to prepare 2′-hydroxy-2,9-dimethyl-6,7-benzomorphan are not complete. The intermediates through the 3-methyl-2(-*p*-methoxybenzyl)-1-methyl-1,2,5,6-tetrahydro pyridine have been characterized. The final step to effect ring closure and O-demethylation is underway. The usual reagents for this procedure failed.

3) Synthesis of methyl-(1,2,3,4-tetra-O-acetyl-β-D-glucopyranosyl)uronate and methyl-(2,3,4-tri-O-acetyl-1-bromo-1-deoxy)-α-D-glucopyranosyl)uronate from glucuronolactone which are to be utilized in synthesizing N- and O-glucuronide derivatives for 6,7-benzomorphans is under way.

Significance: This basic and applied research falls in line with the aims of this Institute and makes available new compounds of pharmacologic interest.

Proposed Course of the Project: To complete the synthesis of the elusive 5-unsubstituted benzomorphan and to modify further, with different functional groups, these benzomorphans. These derivatives are to be screened for biological activity.

Publications: Kanematsu, K., Parfitt, R. T., Jacobson, A. E., Ager, J. H., and May, E. L.: Synthesis of 6,7-Benzomorphan from 4-Phenylpyridine. *J. Am. Chem. Soc.*, 90: 1064 (1968).

Serial No. NIAMD-LC-11
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1) Antigenic Polymers
2) 4-(p-Hydroxyphenyl)-1-vinyl-2-pyrrolidinone
3) Instrumentation

Previous Serial Number: Same

Principal Investigators: T. D. Perrine

Other Investigators: William R. Landis

Cooperating Units: Ira B. Tice (NIH, R-BEI)

Man Years:

Total: 1 1/2
Professional: 1 1/2
Other: 0

Project Description:

Objectives: 1) To investigate the synthesis of polymers with antigenic, antimetabolite and interferon-producing properties. 2) To develop practical methods for the synthesis of title compound 2. 3) To develop new and improved methods of instrumentation.

Major Findings: 1) Previous efforts directed at preparing a ter polymer of maleic anhydride with vinyl pyrrolidone and p-hydroxystyrene were unsuccessful. We have now found that if highly purified p-acetoxystyrene is used in the preparation, and the product is subsequently treated with 2-dimethylaminoethylamine, an amphoteric material containing lactam, amide, amine, carboxyl and phenolic hydroxyl groups is obtained. This material readily couples with diazonium compounds, is water soluble in all pH ranges, and is reversibly denatured upon drying. We are currently attempting to arrange to have this material tested for antigenic and interferon-producing action. Initial attempts to form the glucoside of this polymer have been unsuccessful.

Coupling the above polymer with diazotized arsanilic acid leads to wine-red, water-soluble products. These polymers, with various arsenic contents, are being examined for antigenicity by Dr. Phillip R. B. McMaster of NIAID.

Previously we have reported on amino acid grafts of polyethylenimine. The difficulty of hydrolyzing off the amino acid residues, and the spectral and analytical data suggest that the course of the Leuch's anhydride grafting leads to urea linkages to the polymer. In an effort to overcome this, we have investigated the used of α-azido carboxylic acids. When α-azido β-phenylpropionyl chloride is added to PEI in pyridine solution, an insoluble polymer is obtained. Attempts to reduce this material to phenylalanine-grafted PEI have led to materials with IR absorptions in the 4-5-μ region, and we suspect that extraneous N-N bonds are being formed.

Amino acid-grafted PEI (Leuch's anhydride types) have been sent to Dr. Wm. Regelson for examination as stimulators of interferon production, and to Drs. Lucien A. Bavetta and Marcel E. Nenni for tests in copper chelation in vivo, in relation to their collagen insolubilization studies.

2) Previous routes directed toward the synthesis of title Compound 2 involved the blocking of phenolic hydroxyl with methyl. Two otherwise practical syntheses were developed, but no satisfactory means of removing the methyl was discovered.

In the present work, use of the acetoxyl group is investigated. While this group can be employed, it is too unstable to yield satisfactory yields in all steps of the synthesis, and troublesome phenolic Zwitterions have been encountered which have seriously hampered the analyses and recovery of products. At the present time, the benzyloxy group is being investigated for this purpose. The initial ethyl 4-benzyloxy-α-cyanocinnamate has been obtained in excellent yield.

3) With Ira Tice and Harry Saroff, we have developed a compact and sensitive solid state electrometer for use in Gas Chromatography. Detection sensitivity of this device is probably 10^{-15} mole of carbon.

We have found that for steroids and steroidal alkaloids, copper, monel and inconel columns often give superior results, whereas brass gives almost uniformly bad results. Peak tailing is often a consequence of the metal used for the column. When columns are lined with teflon, results are extremely bad.

With respect to precision viscometry, a satisfactory timing and readout system has been developed. Various methods have been tried for driving the fluid to refill the viscometer. We have found that gas pressure is not a suitable means. It appears that the viscosity of the fluid is strongly affected by the nature of the gas used, and it has not been possible to control condensation and evaporation of the solvent due to the changes of pressure required by the cycle. We are in the process of testing a mechanical piston drive, which looks promising if we can overcome problems associated with volume changes in the system due to the ingress and egress of the rod driving the piston.

Significance: Basic to the biomedical, biochemical and organic chemistry programs of the Institute.

Proposed Course of Projects: 1) Polymers; To further study the amphoteric polymer with a view to adding determinant groups. To study the blocking of PEI in order to minimize cross-linking in amino acid grafting and similar reactions.

2) To examine various reduction proceedures for succinimides with a view to more practical syntheses of title Compound 2.

3) Instrumentation: To examine metallic packings for columns in order to further evaluate the role of the metal in gas chromatographic processess. To develop better means of applying gas chromatography to the mass spectrometric analysis of organic compounds. To continue development of the automatic precision viscometer.

Publication: Perrine, T. D. and Landis, W. R.: Analysis of Polyethylenimine by Spectrophotometry of its Copper Chelate. J. Polymer Sci., Part A-1, Vol. 5, 1993-2003 (1967).

Jarvis, F. G., Mesenko, M. T., Martin, D. G., and Perrine, T. D.: Physicochemical Properties of the Vi Antigen before and after Mild Alkaline Hydrolysis. J. Bacteriol., Vol. 94 #5, 1406-1410 (1967).

Jerina, D., Daly, J., Landis, W., Witkop, B., and Udenfreid, S.: Intramolecular Migration of Tritium and Deuterium during Nonenzymatic Aromatic Hydroxylation. J. Am. Chem. Soc., 89: 3347 (1967).

Cerutti, P., Kondo, Y., Landis, W., and Witkop, B.: Photoreduction of Uridine and Reduction of Dihydrouridine with Sodium Borohydride, J. Am. Chem. Soc., 90: 771 (1968).

Landis, W. R. and Perrine, T. D.: The NMR and IR Spectroscopic Study of N-Vinyl Compounds. Appl. Spectry., in press (1968).

Serial No. NIAMD-LC-12
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Titles: 1) Synthetic approaches to a 2′,5,9-unsubstituted-6,7-benzomorphan.
2) Approaches to new ring systems (e.g. 1,5-methano-2-benzazepine, etc.).
3) Approaches to benzhexahydroazocines.
4) Optical resolution of a 2′-H benzomorphan.
5) Preparation of 2′-halo substituted benzomorphans for anti-inflammatory testing.

Previous Serial Number: Same

Principal Investigators: Arthur E. Jacobson

Other Investigators: M. Takeda and K. Kanematsu (project 1);
M. Mokotoff (project 2)

Cooperating Units: None

Man Years (computed for the 12 month period):

Total: 3/4
Professional: 3/4
Other: 0

Project Description:

Objectives: 1) To find routes to the 2′,5,9-unsubstituted-6,7-benzomorphan.

2) To find general synthetic methods applicable to the formation of 1,5-methano-2-benzazepine, benzazocine, and benzazonine ring systems.

3) To prepare substituted benzhexahydroazocines.

4) To resolve α-2-methyl-5,9-diethyl-6,7-benzomorphan.

5) To prepare α-2′-fluoro- and chloro-2,5,9-trimethylbenzomorphan for anti-inflammatory screening.

Methods Employed: Organic, physical-organic and analytical chemistry and instrumental analysis.

Major Findings: 1) 1-Methyl-1-benzyl-4-hydroxypiperidinium chloride and its tritylether derivative, the supposed 1-methyl-2-benzyl-4-chloro-1,2,5,6-tetrahydropyridine and its associated piperidine derivative, 1-methyl-2-benzylpyridinium iodide, 1-methyl-4-bromopyridinium iodide, and 1-carbethyoxymethylene-3-nitronaphthalene were involved in the various approaches to the 2′,5,9-unsubstituted-6,7-benzomorphan. Each of these routes, for a variety of reasons, failed to give the desired compound. Two other routes which were attempted were successful, and are discussed by E. L. May.

2) A route to 1,5-methano-2-benzazepine is visualized through 3-carbomethoxy-6-methoxy-1-indanone. Various transformations may lead to the desired compound.

3) Neither photolysis of N-chloroacetyl-1-methoxy-3-propylaminobenzene, nor attempted chemical cyclizations of analogous compounds leads to the desired 3-methoxy-benzhexahydro-3-azocine. An approach via the 1-methoxy-4-bromo-N-chloroacetyl substituted compound noted above gave a mixture of products which have not been separated. Other approaches were unsuccessfully attempted and abandoned.

4) α-5,9-Diethyl-6,7-benzomorphan was resolved into its d and l forms using mandelic acid. The salts were too soluble in the medium used, and the yields were poor. Other resolving agents were tried in an attempt to improve the yield of the optical isomers, but they were not as good as the mandelic acid.

5) 2′-Fluoro- and 2′-chloro-2,5,9-trimethyl-6,7-benzomorphan were prepared in quantity for anti-inflammatory testing. They were found to be reasonably active compounds in the various testing procedures tried.

Significance: 1) The unsubstituted benzomorphan has been found to have analgetic activity, a rather surprising finding. It had been postulated that the 5-position must be quaternary for the compound to have analgetic activity.

2) The 1,5-methano-2-benzazepine is, in fact, a β-nor-benzomorphan. The conformation of this molecule may be sufficiently similar to that of the related benzomorphan for it to have analgetic and, perhaps, anti-inflammatory activity.

3) The anti-inflammatory activity of the analgetically weaker benzomorphans is an intriguing finding. There are few, if any, anti-inflammatory compounds known which are basic, rather than acidic (or neutral). These basic benzomorphans must be acting on quite different sites than the known acidic compounds (from aspirin to flufenamic acid). The mechanism of action of such diverse substances is an interesting if, at present, unanswerable question.

Proposed Course of Project: Further work is contemplated for Projects two and five.

Publications: Jacobson, A. E. and Parfitt, R. T.: The Stevens Rearrangement of 1-p-Methoxybenzyl-1,2,5,6-tetrahydropyridinium salts. J. Org. Chem. 32: 1894 (1967).

Kanematsu, K., Parfitt, R. T., Jacobson, A. E., Ager, J. H., and May, E. L.: Synthesis of 6,7-Benzomorphan from 4-Phenylpyridine. J. Am. Chem. Soc. 90: 1064 (1968).

Jacobson, A. E., May, E. L., and Sargent, L. J.: "Analgetics" Chapter 49 in Medicinal Chemistry, 3rd Ed., A. Burger, ed., John Wiley & Sons, Inc., New York. In press 1968.

Serial No. NIAMD-LC-13
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of Synthetic Procedures for Alkaloids

Previous Serial Number: Same

Principal Investigator: Edward M. Fry

Other Investigators: None

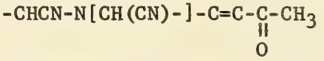
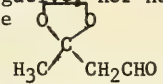
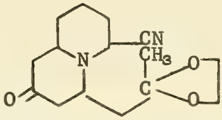
Man Years (computed for the 12 month period):

Total: 1
Professional: 1
Others: 0

Project Description:

Objectives: The synthesis of nitrogen heterocycles.

Methods Employed: Standard methods of organic synthesis and adjuncts thereto.

Major Findings: Attempts to develop an annelation method for the cycl-[3.3.3]azine system have been focused on 2,6-dicyanopiperidine after an initial success in displacing one of the nitrile groups with acetone. Similar efforts to introduce acetone into the  $-CHCN-N[CH(CN)-]-C=C-\underset{\overset{\|}{O}}{C}-CH_3$ (I) system have so far been negative, nor have imino derivatives of I proved useful. At present the aldehyde  which should, under Mannich conditions, readily give  is being investigated.

Significance: Any extension of the knowledge of making heterocyclic nitrogen compounds is of value in the field of synthetic medicinal chemistry.

Publications: None

Serial No. NIAMD-LC-14
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: N-Substituted Derivatives of 5-Methyldihydromorphinone and Desomorphine.

Previous Serial Number: None

Principal Investigators: L. J. Sargent and Everette L. May

Other Investigators: None

Cooperating Units: Pharmacology unit of this Section and the Department of Pharmacology, University of Michigan

Man Years: (computed for the 12 month period)

Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: To replace the N-methyl group of each 5-methyldihydromorphinone (metopon) and dihydrodesoxymorphine-D (desomorphine), two potent morphine-like analgesis, with groups that should impart antagonistic properties and thus reduced dependence liability.

Methods Employed: Usual organic chemical methods including spectrographic techniques.

Major Findings: Both metopon and desomorphine were N-demethylated by the von Braun method or with diethyl azodicarboxylate. The normetopon resulting was converted to the N-phenethyl, N-cyclopropylmethyl and N-3,3-dimethylallylderivatives, and nordesomorphine to the N-cyclopropylmethyl and N-3,3-dimethylallyl derivatives. For the dimethylallyl compounds direct N-alkylation with 3-methyl-3-butene (DMF-$NaHCO_3$) was used while the other derivatives were made by appropriate acylation and LAH reduction.

Nordesomorphine and normetopon were about half as active as morphine in the mouse-hot plate method of assay (s.c. administration), N-phenethylnormetopon is over twice as potent as morphine, and N-cyclopropylmethylnodesomorphine which is expected to be a potent morphine antagonist is between morphine and codeine in analgesic activity.

Significance: Is consonant with the section's goals in providing leads for improved drugs.

Proposed Course of Project: To complete the chemical and analgesic assay work on the above new compounds and to assess them for physical dependence liability and narcotic antagonistic activity in monkeys.

Serial No. NIAMD-LC-15
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunologic studies in clinical Hematology

Previous Serial Number: Same

Principal Investigators: N. Raphael Shulman and Lewis J. Sargent

Other Investigators: None

Cooperating Units: None

Man Years (computed for the 12 month period):

Total: 1/4
Professional: 1/4
Others: 0

Project Description:

Objectives: Determination of the serological specificity of azoproteins derived from 6-aminodihydrocinchonine.

Methods Employed: Dihydroquinine (from the catalytic reduction of quinine) was O-demethylated to dihydrocupreine and the resulting phenolic base converted to 6-aminodihydrocinchonidine (via ammonium bisulfite-sealed tube). Diazotization of the latter was followed by coupling with normal human as well as with certain animal sera and the diazoproteins thus formed examined for antigenic activity.

Significance: A study is being conducted of naturally occurring human antibodies that heretofore had not been shown to react with macromolecular forms of certain drugs. Various antigens are being designed to ascertain whether the antibody reaction can be effected in the absence of cell platelets or cells that are normally required to complete the antigen-antibody reaction.

Proposed course: In view of the ill-defined results obtained in this study, this project has been terminated.

Serial No. NIAMD-LC-16
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Evaluation of Compounds for Analgesic Activity and Dependence Liability

Previous Serial Number: LC-6

Principal Investigator: Everette L. May

Other Investigators: Nathan B. Eddy, Consultant

Cooperating Units: Animal Production Section, NIH; Department of Pharmacology, University of Michigan.

Man Years:

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Objectives: To assess compounds for analgesic activity, dependence liability, and narcotic antagonist activity and to serve in an advisory capacity to the Bureau of Narcotics, FDA, WHO, and the pharmaceutical industry.

Methods Employed: Eddy hot-plate method of analgesic assay and 24-hr. acute toxicity determinations (CDGP mice).

Major Findings: A total of 295 experiments were performed with 90 compounds. These consisted of 80 complete assays for analgesia (most by the subcutaneous, a few by the oral route), 210 dose-range finding experiments, and five acute toxicity assays. The more interesting compounds were sent to the University of Michigan for dependence liability (of the morphine type) determinations. A few of these proved to be powerful pain-relieving drugs with low or no dependence liability in man.

Mrs. Atwell, the technical expert of this facility also continues to assist Drs. Daly and Creveling (Metabolites, LC) with their experiments on biogenic amines and in maintaining a colony of South and Central American dentrobatids.

Significance: Aids the research of the Chemistry units, LC, of the pharmaceutical industry, and of University and other research laboratories engaged in work on CNS type compounds. Also enhances this section's function in an advisory capacity.

Proposed Course of Project: To continue without substantial change.

Honors and Awards:

1. Presented a lecture, "The Chemistry of Deep Analgesics" as a part of a symposium on Deep Analgesics at the Medicinal Chemistry meeting of the Gordon Conferences at Crystal Mountain, Washington, August 1967.

2. Was co-recepient (with Dr. Nathan Eddy) of the 1967 Hillebrand Prize, Chemical Society of Washington.

3. Continued to serve as Consultant to the Executive Secretary of the Committee on Problems of Drug Dependence, NRC, and as a member of WHO Expert committee on Drugs liable to Produce Dependence; the NIGMS, NIH Fellowship Specialty Board; the Chemistry Committee for Malaria, Walter Reed Army Institute of Research, and the CCNSC Contracts Review Committee.

Publications:

Kanematsu, K., Parfitt, R. T., Jacobson, A. E., Ager, J. H., and May, E.L.: Synthesis of 6,7-Benzomorphan from 4-Phenylpyridine. J. Am. Chem. Soc. 90: 1064 (1968).

Jacobson, A. E., May, E. L., and Sargent, L. J.: Analgesics in Medicinal Chemistry, 3rd edition, A. Burger, Ed., Academic Press, in press (1968).

Serial No. NIAMD-LC-17
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Separation of Morphine-like Effects by Optical Resolution

Previous Serial Number: None, although similar work was included under NIAMD-LC-10

Principal Investigator: Everette L. May

Other Investigators: J. Harrison Ager and Arthur E. Jacobson

Cooperating Units: Pharmacology Unit of this Laboratory, and Department of Pharmacology, University of Michigan

Man Years: (computed for the 12 month period)

Total: 3/4
Professional: 3/4
Other: 0

Project Description:

Objectives: To separate harmful (particularly dependence liability) from beneficial neuropharmacologic effects through optical resolution of 6,7-benzomorphans.

Methods Employed: Resolution methods involving, *d*- and *l*-mandelic acids, (+)-3-bromo-8-camphorsulfonic acid, and (+)-10-camphorsulfonic acid. Also pharmacological testing including analgesic and morphine-like dependence liability assays in mice and monkeys, respectively.

Major Findings: Like previously investigated homologs, e.g., α-5,9-diethyl- and α-5-propyl-9-methyl-, (-)-5-ethyl- and 5-methyl-2′-hydroxy-2-methyl-6,7-benzomorphan, analgesics as powerful as morphine, will not support a morphine dependence in monkeys, in fact, are mild nalorphine-like antagonists. The *dextro*-isomers, on the other hand, of a low order of analgesic activity will substitute for morphine in dependent monkeys. These findings are positively consistent in but unique for, the 6,7-benzomorphan class of analgesics and bring us closer to the development of a near-ideal pain-relieving agent.

Significance: Is consonant with the mission of the Institute in providing basic research and new leads for the pharmaceutical industry.

Serial No. NIAMD-LC-18
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Batrachotoxin and Other Physiologically Active Compounds from Amphibian Skins

Previous Serial Number: NIAMD-LC-18

Principal Investigator: Bernhard Witkop

Other Investigators: J. W. Daly, C. R. Creveling and T. Tokuyama

Cooperating Units: Marte Latham, Collector
Charles W. Myers, Herpetologist, Gorgas Memorial Laboratories, Panama
Thomas Turner, Biologist, Univ. of the West Indies, Kingston, Jamaica
Isabella Karle and J. Karle, U. S. Naval Research Laboratory
E. Uyeno, Stanford Research Institute, Stanford, Calif.

Man Years (computed for the 12 month period)

Total: 2
Professional: 1-1/2
Other: 1/2

Project Description:

Objectives: To isolate and purify the highly active poison, batrachotoxin, from extracts of the Colombian arrow poison frog and to elucidate its chemical structure and relate this to its pharmacological effects. To investigate and identify other physiologically active compounds occurring in skins of frogs. To investigate the biosynthesis and pharmacology of dehydrobufotenine and analogs.

Methods Employed: Sensitive bioassay methods, thin layer, column and gas chromatography have been used in the purification of the active principles, while n.m.r., mass spectroscopy, x-ray crystallography, microchemical techniques and radiochemical labeling are being utilized for structure elucidation. Isotopic tracer techniques have been used to study biosynthetic pathways.

Major Findings: The principal steroidal alkaloids occurring in the Colombian poison arrow frog (Phyllobates aurotaenia) have been shown to be batrachotoxin, isobatrachotoxin, pseudobatrachotoxin and batrachotoxinin-A.

Pseudobatrachotoxin has been shown to undergo a rapid hydration to form batrachotoxinin-A. A p-bromobenzoate of batrachotoxinin-A was prepared, crystallized and its structure determined by x-ray crystallographic analysis. The structure, unique among steroids, is that of 3α,9α-epoxy-14β,18β-[epoxyethano-N-methylimino]-5β-pregna-7,16-diene-3β,11α,20α-triol. NMR and mass spectral data indicate similarities between (iso)batrachotoxin and the pseudobatrachotoxin and batrachotoxinin-A.

Another brightly colored frog (Phyllobates vittatus) from Costa Rica has also been found to contain batrachotoxin, isobatrachotoxin and batrachotoxinin-A.

Three toxic steroidal alkaloids from a small brightly colored frog (Dendrobates pumilio) have been isolated and studied by physical techniques.

O-Methylnordehydrobufotenine has been found to effect visual discrimination in squirrel monkeys and is some 2 to 3 times as active as mescaline in this test.

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, third among known toxins, its action on nerve preparations and its cardiotoxic properties gives the elucidation of its structure and correlations of structure with activity a high priority in biomedical research. Other compounds from frog extracts may have physiological activities which will warrant their structural and pharmacological investigation. Examples of compounds of this type, occurring in frogs and toads, include samandarine, a central convulsant, the cardiotoxic bufogenins and bufotoxins, the catecholamines, indolealkylamines and histamines, and recently the hypotensive polypeptides such as bradykinin and physalaemin. Because of its structural similarity to bufotenine and lysergic acid diethylamide, a thorough investigation of the properties of dehydrobufotenine is relevant to fundamental aspects of neurochemistry.

Proposed Course of Project: A crystalline derivative of batrachotoxin or isobatrachotoxin suitable for x-ray crystallographic analysis will be prepared. Biosynthetic studies on the formation of batrachotoxins from serine and cholesterol will be undertaken. Synthesis of related structures will be carried out. Further mass spectral and chemical studies will be done on other compounds isolated from frog extracts. Pharmacological testing on these compounds will be completed. The unique oxidative cyclization of indolealkylamines to dehydrobufotenine will be further studied to demonstrate the immediate precursor and to isolate the enzyme system responsible. Attempts will be made to carry out a chemical or photochemical cyclization of bufotenine to dehydrobufotenine.

Honors and Awards: None

Publications:

Daly, J. W., and Meyers, C. W.: Toxocity of Panamanian Poison Frogs (Dendrobates). Some Chemical and Biological Aspects. Science 156: 970-973, May 1967.

Gannon, W. F., Benigni, J. D., Suzuki, J., and Daly, J. W.: The Synthesis of Dehydrobufotenine. Tetrahedron Letters 16: 1531-1534, Apr. 1967.

Daly, J. W.: Poisons. In McGraw-Hill Yearbook of Science and Technology, 1968, pp. 311-313.

Tokuyama, T., Daly, J. W., Witkop, B., Karle, K., and Karle, J.: The Structure of Batrachotoxinin-A. A Novel Steroidal Alkaloid from the Colombian Arrow Poison Frog, *Phyllobates aurotaenia*. J. Am. Chem. Soc. 90: 1917-1918, 1968.

Daly, J. W., and Witkop, B.: The Venoms of Amphibians. Mem. Inst. Butantan 33: 425-432, 1966.

Serial No. NIAMD-LC-19
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Pharmacodynamic Amines

Previous Serial Number: NIAMD-LC-19

Principal Investigator: John W. Daly

Other Investigators: B. Witkop, C. R. Creveling, H. Ong and T. Tokuyama

Cooperating Units: A. Sjoerdsma, NHI-LET-
J. Axelrod, NIMH-LCS-
Solomon Snyder, Johns Hopkins University

Man Years (computed for the 12 month period)

Total: 1-3/4
Professional: 1-1/2
Other: 1/4

Project Description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indole alkylamines, imidazole alkylamines and their precursors and transformation products. To develop new techniques for the investigation of transport metabolism and binding phenomena as related to these amines and their interactions with various drugs.

Methods Employed: Drugs and substrates labeled with tritium, deuterium or C^{14} have been used to study the metabolic fate of endogenous amines and amino acids and the underlying mechanisms of uptake, release and metabolism of these compounds. New synthetic methods and techniques such as thin-layer and gas chromatography, n.m.r. and mass spectroscopy have been used to facilitate these studies.

Major Findings: The use of the technique of prelabeling cardiac stores of norepinephrine with norepinephrine-^{3}H in order to study the influence of drugs has been applied to structure activity correlations of reserpine and yohimbine derivatives. Amines and amino acids related to the potent norepinephrine releasing agent, 3,5-dihydroxy-4-methoxyphenethylamine, have been studied. The amino acid was found to cause release of norepinephrine and lowered blood pressure in genetically hypertensive rats.

The technique of N-dansylation and mass spectrometry allowed quantitation and identification of a variety of amines from mouse heart and of 3,4-dimethoxyphenethylamine from human urine (schizophrenic).

Tritiated 6-hydroxydopamine was prepared and was found to react *in vivo* irreversibly with various peripheral sympathetically innervated tissues.

Significance to Bio-medical Research and the Program of the Institute: The metabolic fate of peripherally and centrally active biogenic amines is a key problem in modern neurochemistry and psychopharmacology.

Proposed Course of Project: The binding and release of norepinephrine as influenced by drugs will be explored further, as will interactions of certain amines and drugs at storage and receptor sites. All possible trihydroxyphenethylamines will be synthesized and studied since the two known isomers 3,4,5-trihydroxyphenethylamine and 6-hydroxydopamine exhibit remarkable activities with respect to interference with norepinephrine storage. Electron microscopy will be used to determine the subcellular localization of tritiated 6-hydroxydopamine. The amino acid, 6-hydroxydopa, will be synthesized for studies on its effect on brain norepinephrine.

Honors and Awards: None

Publications:

Holmstedt, B., Daly, J. W., Carlos Del pozo, E. C., Isbell, H., and Szara, S. I.: Psycho-Active Action of Various Tryptamine Derivatives. In D. Efron (Ed.): *Ethnopharmacological Search for Psycho-Active Drugs*. Government Printing House, 1967, pp. 374-382.

Creveling, C. R., Daly, J. W., Tokuyama, T., and Witkop, B.: The Combined Use of α-Methyltyrosine Ethyl Ester and *threo*-Dihydroxyphenylserine: Selective Reduction of Dopamine Levels in the Central Nervous System. *Biochem. Pharmacol*. 17: 65-70, 1968.

Creveling, C. R., and Daly, J. W.: Identification of 3,4-Dimethoxyphenethylamine from Schizophrenic Urine by Mass Spectrometry. *Nature* 216: 190-191, 1967.

Creveling, C. R., Daly, J. W., and Witkop, B.: The Chemorelease of Norepinephrine from Mouse Hearts. III. 3,5-Dihydroxy-4-methoxyphenethylamines and Related Compounds. *J. Med. Chem.* 11: 595-596, May 1968.

Creveling, C. R., Daly, J. W., Parfitt, R., and Witkop, B.: The Chemorelease of Norepinephrine from Mouse Hearts. IV. Structure-Activity Relationships. Reserpines and Yohimbines. *J. Med. Chem.* 11: 596-598, May 1968.

Creveling, C. R., Kondo, K., and Daly, J. W.: Use of Dansyl Derivatives and Mass Spectrometry for the Identification of Biogenic Amines. Clin. Chem. 14: 302-309, Apr. 1968.

Axelrod, J., and Daly, J. W.: Phenol-O-methyltransferase. Biochim. Biophys. Acta, in press.

Witkop, B.: The Role of Biochemistry in Drug Design. Am. Scientist 55: 109-128, June 1967; in Japanese: Protein-Nucleic Acid-Enzyme 12: 1026-1037, Oct. 1967.

Serial No. NIAMD-LC-20
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Selective Modification of Free and Bound Tyrosine and Tryptophan

Previous Serial Number: NIAMD-LC-20

Principal Investigator: Thomas F. Spande

Other Investigators: Meir Wilchek, Motonori Ohno and Bernhard Witkop

Cooperating Units: G. W. A. Milne, NHI-LM

Man Years (computed for the 12 month period)

Total: 2-1/4
Professional: 2
Other: 1/4

Project Description:

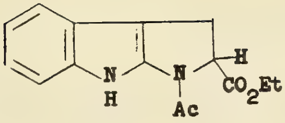
Objectives: To study the oxidation of tyrosine and tryptophan and suitable derivatives thereof, with N-bromosuccinimide, t-butyl hypochlorite, lead tetraacetate and other oxidants. To develop an assay for N-terminal tyrosyl and tryptophyl residues in enzyme digests. To study the reaction product from tryptophan and suitable indole models with the selective reagent 2-hydroxy-5-nitrobenzyl bromide. To devise a reagent suitable for altering the migration rates of tryptophyl peptides on paper electrophoresis. To investigate synthetic routes to certain metabolites containing the indole ring fused to a pyrrolidine ring.

Major Findings: Hydroxy and Dihydroxy Indoles from N-Terminal Tyrosyl Residues: N-terminal tyrosyl peptides, unlike those with an endo-tyrosyl residue, fail to cleave with N-bromosuccinimide. Tyrosine in this environment, undergoes a cyclization reaction to 5,7-dibromo-6-hydroxyindole-2-carboxamido residues, with the production of a characteristic UV absorption at 315 mμ ($\epsilon = 16{,}000$). The cyclization was shown to proceed through an intermediate tribromodienone. When NBS was added at pH 3 and the pH raised to 6-7 shortly thereafter, a dihydroxy indole was produced as the major product (25% yield) with only small amounts (≅ 5%) of the 6-hydroxy indole derivative formed. The dihydroxy indole was identified as ethyl 6-dihydroxy-7-bromoindole-2-carboxylate when the reaction was carried out with ethyl tyrosinate or ethyl 3,5-dibromotyrosinate. The same material resulted when 3,4-dihydroxyphenylalanine ethyl ester (DOPA ethyl ester) was treated with NBS under acidic conditions. In addition, the indoline derivative, ethyl-5,6-dihydroxy-7-bromo-2,3-dihydroindole-2-carboxylate was isolated. This may be an intermediate in this cyclization. Similar derivatives were prepared starting with tyrosinamide and tyrosine-N-methylamide. Thus NBS (or bromine) is capable of converting tyrosyl derivatives with a free amino group to either 6-hydroxy or 5,6-dihydroxyindole derivatives.

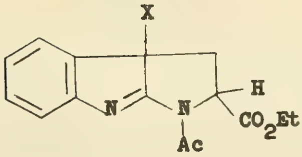
A Rapid Assay of N-terminal Tyrosyl Residues Using a Spectrophotometric Procedure: Existing assays for N-terminal tyrosine (e.g. the fluorodinitrobenzene procedure) are complicated by reaction at both the amino group and the phenolic hydroxyl group. Utilizing the indole chromophore produced when N-terminal tyrosyl residues are exposed to NBS, a procedure was developed to rapidly assay samll amounts (1-2 mg total protein) of enzyme digests for the presence of N-terminal tyrosyl residues. Tryptic digests of glucagon, the globin from myoglobin (sperm whale) and chymotrypsinogen revealed the presence of one, two and three N-terminals of tyrosine, respectively, per mole of protein in agreement with the number of Lys-Tyr or Arg-Tyr sequences in these proteins. The reactions were carried out in 0.1 M formic acid, where an excess of NBS was not disadvantageous to the oxidation-sensitive indole derivative produced.

Alkylation of Tryptophan and Indole Derivatives with 2-Hydroxy-5-nitrobenzyl bromide ("Koshland's reagent"): This reagent, since its introduction in 1964 has been widely applied to label tryptophyl residues in protein studies. At least a dozen papers have appeared featuring this reagent, though the nature of the reaction product remains unknown. Employing the simple indole derivatives, skatole and 2,3-dimethyl indole, M. Wilchek and T. Spande were able to show that, with only a slight excess of reagent, in aqueous acetone, alkylation at the three-position in the pyrrole ring occurs to form initially an indolen ne, which undergoes ring closure with the phenolic hydroxyl group originally on the reagent to form an indoline. The application of the same reaction to N-acetyl tryptamine and N-acetyl-tryptophan ethyl ester led to the isolation of products which had incorporated two moles of reagent. A bis-labelled product was obtained homogeneous from N-Ac tryptamine in 60% yield when two equivalents of reagent were employed. A tentative structure has been advanced based upon NMR, UV, IR and mass spectrometer studies; however, the results of an x-ray study in progress are awaited to settle the structure firmly.

The Alkaline Oxidation of N-blocked, C-blocked Tryptophyl Derivatives: The NBS oxidation of N-acetyl tryptophan esters or amides at pH 8-9 had previously (N. M. Green and B. Witkop: Trans. N. Y. Acad. of Sci. II, 26, p. 659, 1964) been observed to produce a strongly absorbing chromophore at 308 mμ. Isolation proved difficult and was temporarily abandoned. Motonori Ohno was finally successful in isolating good yields (30-40%) of the material responsible for the chromophore, using a very dilute solution of the tryptophan derivative and the rapid addition of exactly one equivalent of NBS. The reaction was shown to be specific for derivatives of tryptophan with both amino and carboxyl blocked. The structure was proved unambiguously by 100 mc NMR and mass spectrometer studies for the oxidation product of N-acetyl-tryptophan ethyl ester, where the unusual tricyclic structure (1) below results.



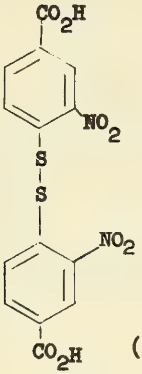
(1)



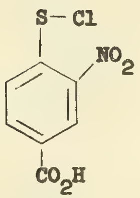
(2) X = Cl, (3) X = OAc

A number of chemical reactions of this material have been studied including a very ready acid hydrolysis to an oxindole, the reduction of the indole double bond with hydrogen or deuterium, and acetylation. The same material can be much more conveniently prepared using t-butyl hypochlorite in a triethylamine buffered $CHCl_3$-CCl_4 solution. A second equivalent converts the indole derivative to a chloroindolenine (2). An acetoxy indolenine (3) results when (1) is treated with lead tetraacetate. Synthetic approaches to the metabolite, Sporidesmin-B, are being currently developed which will exploit these reactions.

2-Nitro-4-carboxy-benzene sulfenylchloride as a Useful Reagent for Visualizing and Isolating Tryptophyl Peptides on High-Voltage Paper Electrophoresis: The reaction of tryptophan with 2-nitrosulfenylchloride has been shown by Scoffone *et al.*, Biochem. Biophys. Res. Commun. 25: 170, 1966, to produce a 2-substituted aryl-thio indole. The reaction is quantitative and can be applied to assay tryptophan in proteins. Employing a reagent slightly modified with a carboxyl group would covalently bind a group to a tryptophyl residue which carries a potential negative charge. This should be useful for detecting and isolating tryptophan-containing peptides using the diagonal electrophoresis technique. Such a procedure would be particularly applicable to the rapid location of tryptophyl peptides in the course of modification studies (e.g. oxidation with NBS) on proteins. The synthetic precursor of the sulfenyl chloride reagent, a disulfide (4) should be useful as a modified Ellman reagent for the detection of thiol groups in proteins.



(4)



(5)

Honors and Awards: None

Publications:

Wilchek, M., Spande, T. F., Witkop, B., and Milne, G. W. A.: Chemical Conversion of Tyrosine to 6-Hydroxyindoles. J. Am. Chem. Soc. 89: 3349-3350, June 1967.

Wilchek, M., Spande, T. F., Milne, G. W. A., and Witkop, B.: The Nonenzymatic Conversion of Tyrosine to Mono- and Dihydroxyindoles. Biochemistry 7: 1777-1786, May 1968.

Wilchek, M., Spande, T. F., and Witkop, B.: A Convenient Spectrophotometric Procedure for the Determination of NH_2-Terminal Tyrosine Residues. Biochemistry 7: 1788-1791, May 1968.

Spande, T. F., Wilchek, M., and Witkop, B.: The Reaction of Derivatives of Tryptophan, Tryptamine, and Other Indoles With 2-Hydroxy-5-nitrobenzyl Bromide (Koshland's Reagent). J. Am. Chem. Soc. 90: 000, June 1968.

Serial No. NIAMD-LC-21
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biosynthesis of Collagen: Inhibitors of Proline Hydroxylase

Previous Serial Number: NIAMD-LC-21

Principal Investigator: Arnold Marglin (Guest Worker from LCB, NHI)

Other Investigators: B. Witkop

Cooperating Units: S. Udenfriend, NHI-LCB-
E. Katz, Georgetown University

Man Years (computed for the 12 month period)

Total: 1-1/4
Professional: 1
Other: 1/4

Project Description:

Objectives: A rapid assay method for proline hydroxylase developed in the laboratory of Dr. S. Udenfriend has made possible a program on selective inhibitors of proline hydroxylase. Proline analogs have been incorporated into oligopeptides.

Methods Employed: Thin layer chromatography, silica gel and ion exchange column chromatography, paper chromatography, and isotope techniques were used for the purification of various intermediates and for the identification of products. Displacement of tosyloxy, mesyloxy, and nisyloxy groups of suitable hydroxyproline derivatives by hydride, deuteride and tritide ion gave selectively labeled and sterically homogeneous L-proline derivatives.

Major Findings: By the use of the Merrifield method peptides varying in chain length from 6 to 12 have been constructed containing the sequence -Gly-Pro-Pro, in which the second or third proline was substituted by analogs and homologs of proline, such as *cis*- and *trans*-4-fluoro-L-proline, *trans*-3-methyl-L-proline, 3,4-dehydroproline and others. Significant inhibitory activity was observed for one hexapeptide, two or three nonapeptides and several dodecapeptides.

The influence of methylproline isomers upon actinomycin biosynthesis was investigated in collaboration with Dr. E. Katz of Georgetown University.

Significance to Bio-medical Research and the Program of the Institute: The mechanism of formation of hydroxyproline, an amino acid found only in the protein collagen, in large amounts, is of biochemical, therapeutic and even clinical interest. Urinary hydroxyproline levels permit to diagnose a number of syndromes and metabolic disorders, such as Marfan's syndrome, chondrosarcomas, acromegaly, etc.

Honors and Awards: None

Publications:

Hutton, J. J., Jr., Marglin, A., Witkop, B., Berger, A., Kurtz, J., and Udenfriend, S.: Synthetic Polypeptides as Substrates and Inhibitors of Collagen Proline Hydroxylase. Arch. Biochem. Biophys., in press.

Serial No. NIAMD-LC-22
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemical Modification of Amino Acids. Selective Nonenzymatic Cleavage of Peptide Bonds. Active Sites of Enzymes. Kinins, Nisin, Subtilin, Pepsin, Peptide Synthesis

Previous Serial Number: NIAMD-LC-22

Principal Investigator: Erhard Gross

Other Investigators: Patricia Q. Lee (through Sept. 1967), Noreen H. Dalgard (from Jan. 1968), John L. Morell and Bernhard Witkop

Cooperating Units: Dr. Marian E. Webster, NHI-LCVP-
Dr. Jack V. Pierce, NHI-LM-
Dr. L. C. Craig, Rockefeller University, New York, N. Y.

Man Years

Total: 2-1/4
Professional: 1
Others: 1-1/4

Project Description:

Objectives: Utilization of reactive groups in polyfunctional amino acids for the purpose of chemical modification. Application of chemical reactions to the selective cleavage of peptide bonds (nonenzymatic fragmentation). Purification of peptide antibiotics and enzymes. Fragmentation and study of residual activity. Inhibition of enzymes and identification of the site of inhibition. Study of environmental factors responsible for enzyme activity and their influence on inhibition. Structural elucidation of peptide antibiotics and structure-function correlations. Peptide synthesis.

Methods Employed: Oxidizing agents for the modification of tryptophan and the cleavage of tryptophyl peptide bonds. Cyanogen bromide for: (a) the cleavage of methionyl peptide bonds; (b) the cleavage of the N-aminoacyl bond of S-alkylcysteine; (c) the removal of the water-soluble amino protecting group acetylmethionine in peptide synthesis; (d) general modification of thioether amino acids. Reduction of β-esters of aspartic acid-containing peptides and cleavage of the resulting homoseryl peptide bond. Different techniques for the synthesis of peptides. Separation and analytical techniques: ion exchange, paper, and thin layer chromatography, gel filtration, countercurrent distribution, electrophoresis, automated ninhydrin analysis, electronic equipment in amino acid analysis, data handling and in process control.

Major Findings: Cyanogen Bromide Reaction: There is virtually no cleavage of methionyllysine at low temperature, a fact that will provide additional peptides in structural studies.

Methylmercaptan may be added to nisin. There is, however, no cleavage of the N-aminoacyl bond of the resulting S-methylcysteine upon treatment with cyanogen bromide.

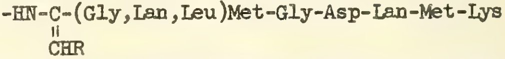
Poorly soluble proteins or insoluble proteins may be solubilized by treatment with cyanogen bromide.

Pepsin-Aspartic Acid: The reactive residue of aspartic acid in pepsin has been converted to the β-ester by treatment with two different inhibitors. Subsequent reduction with lithium borohydride leads to the formation of homoserine lactone and cleavage of the adjacent peptide bond. S-Methylcysteine in reduced (dithioerythritol) and methylated pepsin reacts with cyanogen bromide.

Model peptides with β-esters of aspartic acid and γ-esters of glutamic acid were synthesized for model studies of this nonenzymatic cleavage of aspartyl peptide bonds.

Subtilin: This antibiotic possesses the same carboxyl terminal sequence as nisin, namely dehydroalanyllysine.

Nisin: Nisin may be fragmented in two ways by treatment with cyanogen bromide under different conditions. The cleavage of the methionyllysine bond is suppressed at 0°C. The same peptide bond may be cleaved with cyanogen bromide at 37°C. The various fragments of nisin resulting from the different modes of cleavage have been separated by gel filtration and countercurrent distribution. Three of the five residues of lanthionine in nisin remain with the H_2N-terminal fragment, two are found in the COOH-terminal fragment. The H_2N-terminus of the COOH-terminal fragment is lysine. The following partial sequence may be written for nisin:



$$\text{-HN-}\underset{\underset{\text{CHR}}{\|}}{\text{C}}\text{-(Gly,Lan,Leu)Met-Gly-Asp-Lan-Met-Lys}$$

original NH_2-terminus: isoleucine; original COOH-terminal sequence: dehydroalanyllysine.

There are present in nisin three residues of α,β-unsaturated amino acids, two of these are dehydroalanine, one is β-methyldehydroalanine.

Significance to Bio-medical Research and the Program of the Institute: The cyanogen bromide reaction is widely applied in the structural elucidation of peptides and proteins. Application of the cyanogen bromide reaction to the cleavage of the N-aminoacyl bond of S-methylcysteine contributes to the correct alignment of peptide fragments of proteins. The investigation of enzyme-active sites will be extended to human pepsin(ogen) in a collaborative effort with other activities of the Institute (gastroenterology). The study of human plasma proteins contributes to the understanding of the action of kinins and inflammatory processes. The chemistry of nisin is interesting in several respects: (a) high content of thioether amino acids: lanthionine and methionine;

(b) presence of α,β-unsaturated amino acids; (c) biosynthesis of peptides as opposed to the biosynthesis of proteins; (d) structure-function relationships, e.g., predicted antimalarial activity.

Proposed Course of Project: Continued application of the cyanogen bromide reaction to the fragmentation of peptides and proteins.

a. Plasma - Plasma Proteins - Kininogens - Kinins - Inflammation: Structural elucidation of the active fragments of low molecular weight from human kininogen. Isolation of enzymes and proteins from lysosomes and study of the same for release of peptides with inflammatory properties.

b. Tryptophan: Application of highly selective oxidants to the cleavage of tryptophyl peptide bonds.

c. Methionine: Study of the selective suppression of the cleavage of methionyl peptide bonds with cyanogen bromide.

d. S-Alkylcysteine: Fragmentation of reduced and S-methylated peptides and proteins.

e. Pepsin: Fragmentation of the enzyme at the peptide bonds of methionine, cystine, and the active aspartyl residue. Study of the environment of the active aspartyl residue.

f. Peptide Synthesis: Synthesis of model peptides for the development of new nonenzymatic fragmentation techniques.

g. Cleavage of Aspartyl Peptide Bonds: To be developed to general applicability as nonenzymatic fragmentation technique.

h. Nisin: Continue structural elucidation, desulfurization, primary structure, assignment of the thioether bonds of lanthionines. Biosynthesis. Structure-function relationship. Antimalarial activities. Synthesis of partial sequences-activity studies.

i. Subtilin: Structural studies. Phylogenetic aspects of primary structure (cf. primary structure of nisin).

Honors and Awards: None

Publications:

Gross, E.: The cyanogen bromide reaction. In Hirs, C. H. W. (Ed.): Methods in Enzymology, Vol. 11, Enzyme Structure. New York, N. Y. Academic Press, Inc., 1967, pp. 238-255.

Gross, E.: The Action of Cyanogen Bromide on Protein-Bound Sulfur-Containing Amino Acids. Proceedings, Princeton Conference, The Chemistry of Sulfides, 1966, Princeton, N. J., The Princeton University Press, 1968, pp. 235-256.

Gross, E., Morell, J. L., and Lee, P. Q.: Nonenzymatic Cleavage of Peptide Bonds and Multimolecular Forms of Enzymes. *Ann. N. Y. Acad. Sci.* 149: 000, 1968.

Axen, R., Chaykovsky, M., and Witkop, B.: The Selective Oxidation of Sulfur-Containing Amino Acids by Diethyl Azodicarboxylate. *J. Org. Chem.* 32: 4117-4118, Dec. 1967.

Serial No. NIAMD-LC-23
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Analytical Techniques - Instrumentation
a) Amino Acid Analysis
b) Automatic Monitoring of Column Experiments

Previous Serial Number: NIAMD-LC-23

Principal Investigator: Erhard Gross

Other Investigators: John L. Morell

Cooperating Units: Systems Maintenance Section, DRS-BEI-
Divison of Computer Research and Technology

Man Years (computed for the 12 month period)

Total: 1-1/4
Professional: 1
Other: 1/4

Project Description:

Objectives: Determination of the amino acid composition of peptides and proteins, advanced automation of existing analytical instruments and such techniques which require many time-consuming operations. Adaptation of existing amino acid analyzers to the separation of unusual amine acids. Installation of a multiple sample applicator, to be followed by an automatic sample applicator of sufficient sample capacity to assure continuous operation for a 24-hour period without attendance.

Methods Employed: Ion exchange chromatography, custom resins of spherical shape, colorimeters, recorders and electronic data processing equipment.

1. Automatic Integration: Two electronic integrators have been used extensively in the calculation of the amino acid content of peptide and protein hydrolysates.

2. Automatic Column Regeneration: One analyzer is fully equipped for automatic column regeneration immediately after completion of an analysis and can prepare the column for essentially continuous operation.

3. Automatic Sample Application: A multiple sample applicator of manual operation is the prototype of the Automatic Sample Applicator. The Multiple Sample Applicator accepts four samples at one time. Semicontinuous operation is possible by merely advancing the next sample into the buffer supply line to the column. The construction of the Automatic Sample Applicator in partial collaboration with the Biomedical Engineering Section of the Division of Research Services has now been built and is presently being tested. The final version of automatic sample application calls for the use of two columns and alternate sample admission.

4. Automation of ninhydrin analysis of column effluents has been improved. The existing "robot chemist" is to be replaced by a redesigned model.

5. April 1, 1967 through March 31, 1968: Total number of amino acid analyses: 895.

Significance to Bio-medical Research and the Program of the Institute: The study of the primary structure of peptides and proteins requires large numbers of amino acids. The extensive use of amino acid analytical equipment has significantly contributed to the structural elucidation of peptide antibiotics, e.g. nisin, for which the natural occurrence of dehydroalanine was demonstrated for the first time by the detection and identification of pyruvyllysine on an amino acid analyzer column.

The laboratory has continued to provide institute-wide amino acid analytical services.

Proposed Course of Project: The Automatic Sample Applicator will be tested and modified as the requirements may be. A second one will be built for the final stage of automatic sample application. The amino acid analyzer with automatic sample applicator will be linked to a small computer for the purpose of demonstrating feedback and process control.

The hydrolysis of peptides and proteins will be automated and samples will reach the ion exchange columns directly via the automatic sample applicator.

Further automation of laboratory processes in collaboration with the Division of Computer Research and Technology.

Sequence determination of peptides and proteins a) by utilizing solid supports and b) by employing mass spectroscopic techniques.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-24
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Photochemistry of Free and Bound Nucleotides

Previous Serial Number: NIAMD-LC-24

Principal Investigator: Y. Kondo and T. Kunieda

Other Investigators: B. Witkop

Cooperating Units: Professor Lawrence Gressman, Brandeis University
Prefessor S. Wang, Johns Hopkins University

Man Years (computed for the 12 month period)

Total: 1-3/4
Professional: 1-1/2
Other: 1/4

Project Description:

Objectives: For the sequence determination as well as the understanding of mutagenesis, there is need for highly selective agents which modify selectively single building stones of biopolymers. The photolysis of dihydrothymidine, in contrast to dihydrouridine, leads to selective photodecarboxylation which is demonstrated for the first time for a heterocyclic representative of biological significance.

The mechanism of this photolysis is being studied with dihydrothymine and 1,3-dimethyldihydrothymine in deuterium oxide.

Methods Employed: UV irradiation, thin-layer and paper chromatography, ion exchange resin column chromatography, ultraviolet, infrared, nuclear magnetic resonance, and mass spectroscopy.

Major Findings: Irradiation of an aqueous unbuffered solution of 1-dihydrothymidine for 27-30 hr with a high pressure mercury lamp leads to complete disappearance of the UV absorption at 230 mμ. Purification by column chromatography led to isolation of N_1-deoxyribosyl-N_1-*n*-propylurea, *n*-propylurea and (S)-dihydrothymine. Dihydrothymine on photolysis gave *n*-propylurea and urea. Under identical conditions the photolysis of dihydrouridine led to minor cleavage of the ribosyl residue and to the isolation of 5% of dihydrouracil.

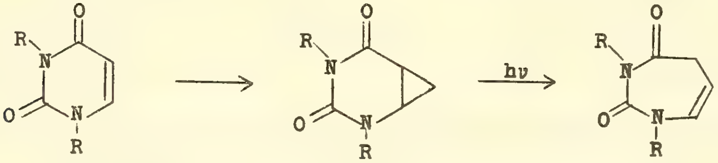
Analogous photolytic experiments were extended to hydantoin derivatives such as hydantoin, 5,5-dimethylhydantoin, hydantoin-5-(acetic acid) and allantoin, which possess similar partial structures related to dihydropyrimidine bases.

Thymine isomers, II, containing a three-membered ring, are of considerable interest not only chemically, but also biologically. They were first synthesized by the reaction of 1,3-disubstituted uracils with dimethyloxosulfonium methylide in good yields.

These isomers (R = Me, $C_6H_5CH_2^-$) are very sensitive to UV irradiation and easily converted to seven-membered rings, III, which are also unstable.

These isomers resist catalytic hydrogenation and afford 1,3-disubstituted urea and not uracil derivatives on acidic hydrolysis.

In all cases, expected photoproducts, ureido derivatives and urea, can be isolated in good yield.



I II III

Honors and Awards: None

Publications:

Kondo, Y., and Witkop, B.: The Selective Photolysis of Dihydrothymidine. J. Am. Chem. Soc. 90: 000, June 1968.

Witkop, B.: Mechanisms of Photoreductions and Hydrogenolysis of Pyrimidine Nucleosides and Their Photodimers. Photochemistry and Photobiology 7, 000, June 1968.

Kondo, Y. and Witkop, B.: Reductive Ring Openings of Glutarimides and Barbiturates with Sodium Borohydride. J. Org. Chem. 33: 206-212, Jan. 1968.

Kondo, Y. and Witkop, B.: The Stereochemistry of the Catalytic and Light-Induced Reduction of Thymidine to Dihydrothymidine and Ureidoalcohols. J. Am. Chem. Soc. 90: 764-770, Jan. 1968.

Kunieda, T., and Witkop, B.: Hydrogenolysis of Thymine Dimer to Cyclobutanes by Sodium Borohydride. J. Am. Chem. Soc. 89: 4232-4233, Aug. 1967.

Cerutti, P., Kondo. Y., Landis, W., and Witkop, B.: Photoreduction of Uridine and Reduction of Dihydrouridine with Sodium Borohydride. J. Am. Chem. Soc. 90: 771-775, Jan. 1968.

Serial No. NIAMD-LC-25
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Reductive Photochemistry of Free and Bound (Aromatic) Amino Acids and Amines

Previous Serial Number: NIAMD-LC-25

Principal Investigator: Bernhard Witkop

Other Investigators: T. Spande, C. Foltz, and T. Tokuyama

Cooperating Units: O. Yonemitsu, University of Hokkaido, Sapporo, Japan

Man Years (computed for the 12 month period)

Total: 2-1/4
Professional: 2
Other: 1/4

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins are needed for the study of the composition and function of their active sites. Although extensive interest to develop such reactions has been shown by many investigators, photochemical methods were applied only recently to this problem. Photochemical methods have been elaborated which make it possible to deliver selectively a distinct amount of energy to a system. The photochemical behavior of free and bound aromatic amino acids and the corresponding pharmacodynamic amines and certain of their derivatives therefore has been investigated.

Methods Employed: Ultraviolet irradiation, thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: Ultraviolet irradiation of aqueous solutions of L-tryptophan in the presence of excess sodium borohydride leads to 4,7-dihydro-L-tryptophan (10%), 2,3-dihydro-L-tryptophan (4%), indole-3-propionic acid (2%) and a trace of tryptamine. Ultraviolet irradiation converts N-chloro-acetyl-L-tryptophan into the eight-membered lactam of L-tryptophan-4-acetic acid. Yields of up to 40% are obtained when the photoinduced cyclization is carried out in neutral aqueous solution (Yonemitsu, O., Cerutti, P., and Witkop, B.: Photoreductions and Photocyclizations of Tryptophan. J. Am. Chem. Soc. 88: 3941-3945, Sept. 1966).

The photocyclization of N-chloroacetyl-L-tryptophan provides convenient access to tricyclic derivatives of indole. The scope of this reaction has been investigated by applying it to other aromatic amino acids and pharmacodynamic amines.

In the photocyclization of N-chloroacetyl-L-tryptophan the original chromophore remains unchanged. However, in the case of N-chloroacetyl-L-tyrosine irradiation in aqueous solution under nitrogen resulted in rapid disappearance of the phenolic chromophore. Irradiation of the homologous O-methyl-N-chloroacetyl-L-tyrosine led to a slower disappearance of the anisole chromophore and the concomitant appearance of a chromophore with λmax 352 mμ. This product was characterized as its methyl ester. Mass spectrometry confirmed the composition of this ester as $C_{12}H_{13}NO_4$. The structure of this product of a deep-seated photolytic rearrangement was determined by x-ray crystallography to be L-5-carbomethoxy-7-formyl-1,2,5,6-tetrahydro-3H-pyrrolo[1,2-A]azepin-3-one. A similar rearrangement takes place on UV-irradiation of N-chloroacetyl-p-O-methyltyramine to produce 7-formyl-1,2,5,6-tetrahydro-3H-pyrrolo[1,2-A]azepin-3-one.

N-Chloroacetyl-m-tyramine and N-chloroacetyl-m-tyrosine on irradiation in aqueous solution are cyclized without rearrangement of 7-hydroxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one and its 4-carboxy derivative. These compounds were methylated to the 7-methoxy- and 7-methoxy-4-carbomethoxy derivatives, respectively. The 7-methoxy lactam was reduced by diborane in tetrahydrofuran to the amine, 7-methoxy-1,2,4,5-tetrahydro-3H-3-benzazepine.

N-Chloroacetyl-3,4-dihydroxyphenethylamine undergoes photocyclization with ring closure into the positions para and ortho to the meta-hydroxy groups. After acetylation the crystalline photocyclization products were N-acetyl-7,8-diacetoxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one and N-acetyl-8,9-diacetoxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one.

N-Chloroacetyl-3-hydroxy-4,5-dimethoxyphenethylamine, a derivative of normescaline, on irradiation gave a single product, the anhalonidine analog 7,8-dimethoxy-9-hydroxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one, resulting from photocyclization into the position ortho to the phenolic hydroxyl. N-Chloroacetyl-N-bromoacetyl- and N-iodoacetylmescaline in preliminary photocyclization attempts were converted to N-acetylmescaline. N-Chloroacetyl-3,5-dibromotyrosine on irradiation gave no identifiable products.

Ultraviolet irradiation of N-chloroacetyltryptamine produced rapid cyclization (4×10^{-3} mole was 95% transformed in 64 min) to the eight-membered lactam, 6-oxo-3,4,6,7-tetrahydro-1H,5H-azocin[4,5,6-c,d]indole, mp 314-315° (dec). The yield of crude product is >90%. Irradiation of N-chloroacetylbromotryptamine yielded 24% of the nine-membered lactam, 7-oxo-3,4,5,6,7-hexahydro-1H,6H-azonin[4,5,6-c,d]indole, mp 279-281°. It is hoped that irradiation of N-chloroacetyl-3-(4'-aminobutyl)indole will afford a practical route to the ten-membered lactam, 8-oxo-3,4,5,6,8,9-hexahydro-1H,7H-azecin[4,5,6-c,d]indole. In preliminary experiments small yeilds of high melting product have been obtained.

Significance to Biomedical Research and the Program of the Institute: The selective modification of single amino acids in proteins permits correlation between tertiary structure and reactivity, yields information on groups close to, or involved in, active sites of enzymes, and makes possible the formation of covalent bonds between reactive amino acid units in proteins. The application of photochemical methods to aromatic amino acids and related pharmacodynamic amines and their derivatives affords new compounds and new routes to compounds of biological interest.

Honors and Awards: None

Publications:

Yonemitsu, O., Tokuyama, T., Chaykovsky, M., and Witkop, B.: Photocyclizations of Tyrosines, Tyramines, Catecholamines, and Normescaline. J. Am. Chem. Soc. 90: 776-784, Jan. 1968.

Serial No. NIAMD-LC-26
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Proline Analogs and Their Effects upon the Biosynthesis of Actinomycin, and Chemical Modification of Actinomycin

Previous Serial Number: NIAMD-LC-26

Principal Investigator: Bernhard Witkop

Other Investigators: None

Cooperating Units: E. Katz, Dept. of Microbiology, Georgetown University
A. B. Mauger, Washington Hospital Center

Man Years

Total: 0
Professional: 0
Others: 0

The work on this project has been completed.

Serial No. NIAMD-LC-27
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Reactivity of Tryptophan in Enzymes as a Function of Protein Environment; Chemical Approaches to the Selective Modification of Tryptophan in Proteins

Previous Serial Number: NIAMD-LC-16

Principal Investigator: T. F. Spande

Other Investigators: B. Witkop

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Others: 0

The work on this project has been completed, and certain aspects of this work have been recently reviewed.

Honors and Awards: None

Publications:

Ramachandran, L. K., and Witkop, B.: N-Bromosuccinimide Cleavage of Peptides. In Hirs, C. H. W. (Ed.): Methods in Enzymology, vol. 11. New York, N. Y., Academic Press, Inc., 1967, pp. 283-299.

Witkop, B.: Miscellaneous Nonenzymatic Cleavages of the Peptide Bond. In Hirs, C. H. W. (Ed.): Methods in Enzymology, vol. 11. New York, N. Y., Academic Press, Inc., 1967, pp. 308-315.

Spande, T. F., and Witkop, B.: Determination of the Tryptophan Content of Proteins with N-Bromosuccinimide. In Hirs, C. H. W. (Ed.): Methods in Enzymology, vol. 11. New York, N. Y., Academic Press, Inc., 1967, pp. 498-506.

Spande, T. F., and Witkop, B.: Tryptophan Involvement in the Function of Enzymes and Protein Hormones as Determined by Selective Oxidation with N-Bromosuccinimide. In Hirs, C. H. W. (Ed.): Methods in Enzymology, vol. 11. New York, N. Y., Academic Press, Inc., 1967, pp. 506-522.

Spande, T. F., and Witkop, B.: Tryptophan Involvement in Binding Sites of Proteins and in Enzyme-Inhibitor Complexes as Determined by Oxidation with N-Bromosuccinimide. In Hirs, C. H. W. (Ed.): Methods in Enzymology, vol. 11. New York, N. Y., Academic Press, Inc., 1967, pp. 522-527.

Spande, T. F., and Witkop, B.: Reactivity Toward N-Bromosuccinimide as a Criterion for Buried and Exposed Tryptophan Residues in Proteins. In Hirs, C. H. W. (Ed.): Methods in Enzymology, vol. 11. New York, N. Y., Academic Press, Inc., 1967, pp. 528-532.

Serial No. NIAMD-LC-28
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Biosynthetic and Catabolic Enzymes Concerned With Biogenic Amines

Previous Serial Number: NIAMD-LC-17

Principal Investigator: Cyrus R. Creveling

Other Investigators: B. Nikodijevic, J. Daly, T. Kunieda, and D. Jerina

Cooperating Units: None

Man Years (computed for the 12 month period)

Total: 2-3/4
Professional: 2-1/2
Other: 1/4

Project Description:

Objectives: To isolate and purify enzymes operative in the biosynthesis and metabolism of biogenic amines. To determine the physical properties of the purified enzymes; sedimentation constants, ratio of axis, molecular weight, amino acid composition, primary structure and the occurrence of isozymic structures. To investigate such enzymatic properties of these enzymes as the effect of pH, temperature, substrate specificity, reversible and irreversible inhibitors and the ionic composition of the incubation media. To develop assays and to apply these enzymatic assays to determine specific biogenic amines and to study the distribution and variation of enzyme levels.

Methods Employed: Standard methods of enzyme purification including: differential centrifugation, density gradient centrifugation, gel filtration, electrophoresis, standard column chromatography and column chromatography using cellulose specifically charged with substrates. Radiochemical and spectrophotometric assays. Preparation and application of specifically labeled deuterated and tritiated substrates for the development of assays and for studies on mechanism.

Major Findings: A modified method for the purification of catechol-O-methyltransferase has been developed. The purified enzyme has been used to develop a sensitive assay for nanogram quantities of norepinephrine. A sensitive enzyme assay, using dihydroxybenzoic acid as substrate, has been developed and used to study the species variation, anatomic and intracellular distribution and induction of this enzyme *in vivo* by such agents as phenobarbital and benzypyrene. A convenient, nonisotopic, spectrophotometric assay, using 4-nitrocatechol as substrate, has been developed. This assay has been used to study the kinetics of enzyme inhibition and to provide a simple screen for inhibitors of this enzyme.

The anatomic and intracellular distribution of tyrosine hydroxylase, dopa decarboxylase and dopamine-β-hydroxylase in the canine brain has been determined.

A microradiochemical assay for dopamine-β-hydroxylase has been developed. β,β-Dideuterotyramine and α,α-dideuterotyramine have been synthesized to confirm a primary isotope effect observed with the corresponding β,β-ditritio compound.

Significance to Biomedical Research and the Program of the Institute: The biosynthesis and metabolism of biogenic amines are of fundamental importance to the fields of neurochemistry, pharmacology and have a direct bearing on the understanding of the functioning of the cardiovascular system.

Proposed Course of Project: Studies on the physical properties of catechol-O-methyltransferase will be continued. Studies of inhibitors of tyrosine hydroxylase, dopamine-β-hydroxylase and catechol-O-methyltransferase will be continued. The use of COMT for the assay of norepinephrine, with special emphasis on the assay of plasma norepinephrine, will be continued. A new area, the induction of COMT in rodent liver, heart and brain will be expanded, based on positive findings with phenobarbital-induced increases in this enzyme.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-29
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Mechanism of Enzymatic Hydroxylation

Previous Serial Number: NIAMD-LC-19

Principal Investigator: John W. Daly

Other Investigators: B. Witkop and D. Jerina

Cooperating Units: S. Udenfriend, NHI-LCB-
G. Guroff, NHI-LCB-

Man Years (computed for the 12 month period)

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

Objective: To study the basic mechanism of oxygen activation and enzymatic incorporation into organic substrates.

Methods Employed: Isotopically labeled substrates, water and oxygen gas, have been used to study the oxidation of organic compounds with a variety of enzymes. Nuclear magnetic resonance spectroscopy, mass spectrometry and other physico-chemical techniques have been employed frequently in these studies.

Major Findings: The mechanism underlying the hydroxylation-induced migration of substituents during aryl hydroxylation ("The NIH Shift") has been further delineated. The degree of migration and retention of deuterium and tritium has been studied in relation to other ring substituents. The results allow the separation of substrates for aryl hydroxylases into two classes; one class comprises nitrobenzene, halobenzenes, phenylethers, biphenyls, cyanobenzene, etc., which do not have an ionizable substituent and the other class such as phenols, anilides and aniline which have an ionizable substituent which can stabilize a cationoid intermediate by ionization rather than by the intramolecular migration of isotopic hydrogen. During these studies the reactivity of substrates of microsomal hydroxylase was found to correlate well to the relative reactivity of the substrate to electrophilic substitution.

The degree of migration and retention of deuterium with acetanilide-4-^{2}H was found to be effected by induction of microsomal enzymes with either phenobarbital or benzpyrene.

Further examples of group migrations for alkyl, chloro, bromo and trifluoromethyl substituents have been demonstrated. Thus, hydroxylation of 4-methylphenylalanine with phenylalanine hydroxylase gives rise to 3-methyltyrosine. Surprisingly, the other product formed with this enzyme is 4-hydroxymethylphenylalanine. This represents the first clear instance of an aliphatic hydroxylation catalyzed by an aryl hydroxylase, in this case phenylalanine hydroxylase.

Various arene oxides, often suggested as intermediates in aryl hydroxylation and in the formation of dihydrodiols and premercapturic acids, have been synthesized. These were benzene oxide, 4-deuterotoluene-3,4-oxide and napthalene oxide. Benzene oxide, *in vitro* with liver preparations, gives rise to phenol, benzenedihydrodiol and a premercapturic acid. The phenol is formed nonenzymatically while the other products are formed respectively by a specific epoxide hydrase and a glutathione-S-epoxide transferase. Direct evidence for the unstable benzene oxide as an intermediate could not, however, be obtained. The 4-deutero-toluene-3,4-oxide rearranges to 3-deutero-4-hydroxytoluene proving that arene oxides are also compatible with the "NIH Shift". Oxygen-18 studies with tyrosine hydroxylase are also compatible with the postulate of an intermediate 3,4-oxide.

Studies on model hydroxylating systems indicate that there are at present no completely satisfactory models for enzymatic hydroxylation.

Significance to Biomedical Research and the Program of the Institute: Enzymatic oxidation reactions are extremely important in many biosynthetic pathways and in the metabolism, detoxication and action of most drugs.

Proposed Course of Project: The mechanism(s) of enzymatic hydroxylation will be further investigated. Arene epoxides of other aromatic substrates will be synthesized and attempts will be made, using tracer technology, to demonstrate their intermediacy in aryl oxidation. The mechanism of aliphatic hydroxylation by aryl hydroxylase will be investigated with oxygen-18. The mechanism by which phenobarbital and benzpyrene effect the "NIH Shift" will be investigated and attempts will be made to purify the enzymes involved. Further attempts to develop satisfactory model systems will be made.

Honors and Awards: None

Publications:

Daly, J. W., Guroff, G., Udenfriend, S., and Witkop, B.: Hydroxylation-induced Migrations of Tritium in Several Substrates of Liver Aryl Hydroxylases. *Arch. Biochem. Biophys.* 122: 218-223, Oct. 1967.

Guroff, G., and Daly, J. W.: Quantitative Studies on the Hydroxylation-Induced Migration of Deuterium and Tritium During Phenylalanine Hydroxylation. *Arch. Biochem. Biophys.* 122: 212-217, 1967.

Daly, J. W., Guroff, G., Udenfriend, S., and Witkop, B.: Hydroxylation of Alkyl and Halogen Substituted Anilines and Acetanilides by Microsomal Hydroxylases. Biochem. Pharmacol. 17: 31-37, 1968.

Daly, J. W., Guroff, G., Jerina, D., Udenfriend, S., and Witkop, B.: Intramolecular Migrations During Hydroxylation of Aromatic Compounds: The NIH Shift. Advances in Chemistry Series, American Chemical Society, in press.

Jerina, D., Daly, J. W., and Witkop, B.: Deuterium Migration During the Acid-Catalyzed Dehydration of 6-Deutero-5,6-dihydroxy-3-chloro-1,3-cyclohexadiene, A Nonenzymatic Model for the NIH Shift. J. Am. Chem. Soc. 89: 5488-5489, 1967.

Daly, J. W., Levitt, M., Guroff, G., and Udenfriend, S.: Isotope Studies on the Mechanism of Action of Adrenal Tyrosine Hydroxylase. Arch. Biochem. Biophys., in press.

Daly, J. W., and Guroff, G.: The Production of m-Methyltyrosine and p-Hydroxymethylphenylalanine from p-Methylphenylalanine Hydroxylase. Arch. Biochem. Biophys., in press.

Jerina, D., Guroff, G., and Daly, J. W.: Enzymatic and Nonenzymatic Hydroxylation and Chlorination of p-Deuteroanisole. Arch. Biochem. Biophys. 124: 612-615, 1968.

Daly, J. W.: The Hydroxylation-Induced Migration of Tritium: Theoretical Aspects. Advances in Tracer Methodology, in press.

Udenfriend, S., Guroff, G., Daly, J. W., Jerina, D., Witkop, B., and Zaltzman-Nirenberg, P. Significance of the NIH Shift With Respect to Liver Microsome Hydroxylations. Proceedings of the International Symposium on Microsomes and Drug Oxidation, Academic Press, 1968, in press.

Serial No. NIAMD-LC-30
1. Chemistry
2. Microanalytical Services and Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Section on Microanalytical Services and Instrumentation

Previous Serial Number: NIAMD-LC-27

Principal Investigators: W. C. Alford and H. K. Miller

Other Investigators: Anne Wright, Josephine Goodwin (6 months), William R. Landis (6 months), Benjamin Miller, Paula Parisius, Byron Baer, Alice Wong, Evelyn Peake

Cooperating Units: None

Man Years

Total: 8-3/4
Professional: 6-1/2
Other: 2-1/4

Project Description:

The Section on Microanalytical Services and Instrumentation is a service organization which provides instrumental and chemical analyses to approximately 150 research scientists at NIH and to a limited extent to personnel of other government agencies including: Department of Agriculture, Food and Drug Administration, Naval Medical Research Institute, and U. S. Public Health Service hospitals. About 30 of the more common elements and 10 or more functional groups are routinely determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by the Rast-camphor method but more often by vapor pressure osmometry in both aqueous and nonaqueous solvents and by mass spectrometry. Instrumental analyses include: mass spectrometry, infrared, nuclear magnetic resonance, ultraviolet, optical rotation, optical rotatory dispersion, and flame photometer. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colorimetric, gasometric, coulometric, potentiometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses remained about constant at around 14,000. These include: carbon-1900, hydrogen-1900, nitrogen-3600 (divided almost equally between Dumas, Kjeldahl and Nessler), phosphorus-200, sulfur-250, halogens-400, miscellaneous functional groups-100, metals-150, weight loss-500, miscellaneous-200, infrared spectra-900, nuclear magnetic resonance spectra-3400, mass spectra-400. In addition several hundred instrumental analyses have been performed by individual research workers who use our equipment in the evenings or on week ends.

The principal investigators spend much of their time in trouble shooting on various pieces of equipment and in consultation with research personnel concerning nonroutine problems which often require special techniques. Mr. Miller has spent several weeks in an attempt to elucidate the structure and/or reaction mechanism of an amide prepared by Mr. W. Landis and Mr. T. Perrine. He also did an extensive study of bonding characteristics on materials prepared by Dr. A. Jacobson. A growing demand for nitrogen determinations in the ultra-micro range (0-10 γ N) prompted the development of a technique whereby such amounts can be routinely determined to an accuracy of about ± 0.5 microgram of nitrogen. This has proved quite valuable to investigators who work with submilligram amounts of enzymes or other hard-to-get proteins.

A useful ancillary function of the Section is the provision of personnel (Mr. Benjamin Miller) to maintain an extensive stock of organic chemicals. This stockroom is for the use of NIAMD chemists primarily but is also open to scientists of other Institutes since there is no similar stock of organics elsewhere at NIH. Mr. Miller also maintains a stock of inorganic chemicals, glassware, compressed gases, shop tools and a small glass blowing facility. As time permits he performs simple to moderately difficult glass blowing jobs for research personnel.

The Section Chief serves as Project Officer on Contract PH 43-65-32 between NIAMD and the Medical Research Council of England. This contract is for the partial support of a program which provides reference steroid compounds to qualified research workers in the United States as well as to a few foreign investigators. More than 600 compounds are available and small amounts (0.5 mg) are provided without cost for use as reference standards. Most of these materials are not available elsewhere in a comparable state of purity.

In February, 1968 a high resolution, double focusing Hitachi Mass Spectrometer was placed in service. This instrument is proving to be very satisfactory in meeting a long-felt need for both routine low resolution mass spectra and non-routine high resolution spectra. The peak matching accessory for determining precise masses is usable but is not as efficient as had been hoped. At some future time it may be desirable to add a small computer to the mass spectrometer in order to do "element mapping" more rapidly and efficiently. Such instrumentation is presently being put into service in several laboratories around the country and we expect to delay consideration of purchase until it is thoroughly proven in service.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-31
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Degradation and Structural Elucidation of the Triterpenoid, Carpesterol

Previous Serial No.: NIAMD-LC-30

Principal Investigator: John A. Beisler

Other Investigator: Yoshio Sato

Cooperating Unit: David L. Rogerson, NIAMD-LNE

Man Years (computed for the 12 month period)
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: The lipid fraction of Solanum xanthocarpum yields a triterpene of unknown structure. The result sought is the complete chemical and structural elucidation of the triterpene using both chemical and physical techniques.

Methods Employed and Major Findings: Carpesterol is a tetracyclic triterpene having a trisubstituted enone chromophore and two hydroxyl groups of which one is esterified as a benzoate. Oxidation of the free hydroxyl function gave a ketone which had a similar RD curve to carpesterol suggesting that the hydroxyl is on the isooctyl-sidechain. The mass spectrum of the ketone indicated that the parent hydroxyl is located at the C_{23}-position. Reduction of carpesterol tosylate with $LiAlH_4$ effects the elimination of the sulfonyl-ester group with the formation of an olefin. The olefin was converted to a glycol in three stages that produced a mass spectrum consistent with a 23,24-dihydroxy-compound.

Conversion of the α,β-unsaturated ketonic system of carpesterol to the homo- and heteroannular dienes provided only minimal information.

Systematic replacement of the oxygen in carpesterol with hydrogen, excepting the oxygen attached to the 3-position, led to a series of derivatives that were non-identical to the corresponding series of the lanosterol, euphol, tirucallenol, dammaranol, and 4-methylfusidanol groups. Thus, a unique tetracyclic triterpenoid skeleton is manifest.

Significance to Biochemical Research and the Program of the Institute: The steroidal alkaloid, solasodine, isolable from S. xanthocarpum, could occur via a biogenetic pathway that might include carpesterol as one of its precursors. This speculation requires a detailed knowledge of carpesterol to stimulate further thought on the biogenesis of solasodine.

Proposed Course of Project: Chemical methods will continue to be used to establish the structure of carpesterol with the advent of a large supply of the natural product from a pilot plant scale extraction. In addition, suitable crystalline derivatives are being synthesized with the intent of applying x-ray cyrstallographic analysis.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-32
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The von Braun Degradation of Demissidine

Previous Serial No.: None

Principal Investigator: John A. Beisler

Other Investigator: Yoshio Sato

Cooperating Unit: None

Man Years (computed for the 12 month period)
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objective: The well known steroidal alkaloid, solanidine, and its dihydroderivative, demissidine (also a natural product) have been resistant to degradation in the vicinity of the tertiary nitrogen atom. Renewed attempts were planned to cleave the nitrogen atom, in part, from the compact fused ring system.

Methods Employed and Major Findings: Although earlier workers unsuccessfully attempted a von Braun degradation of solanidine with cyanogen bromide, a bromocyanamide was obtained in good yield from demissidine acetate. The IR and NMR spectra were in accord with a von Braun product. Reduction of the bromocyanamide with $LiAlH_4$ gave as the major product (20S:22R:25S)-22,26 imino-5α-cholestan-3β-ol which was identified by comparison of the melting point, specific rotation, and the IR spectrum with published values, and by the appearance of an intense peak at m/e 98 in the mass spectrum. Demissidine was also formed as a minor product of the reduction, presumably by displacement of the 17α-bromine atom by the reduced nitrogen.

Significance to Biochemical Research and the Program of the Institute: The steroidal amines which can be generated by the von Braun degradation of demissidine acetate provide hopeful subjects for anti-tumor and antihypertensive evaluation.

Proposed Course of Project: A complete study of the bromocyanamide will be undertaken with a view toward developing it as a key synthetic intermediate (e.g., solacongestidine).

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-33
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Study of the Alkaloids from Solanum congestiflorum (Natri)

Previous Serial No.: NIAMD-LC-29

Principal Investigator: Hidesato Kataoka

Other Investigator: Yoshio Sato

Cooperating Unit: None

Man Years (computed for the 12 month period)
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: To ascertain the chemical structure of alkaloids present in Solanum congestiflorum for possible conversion into useful substances and to determine their biogenetic relationship with other Solanum species.

Methods Employed and Major Findings: The structures of the four alkamines isolated from Solanum congestiflorum previously were based heavily on spectrochemical evidence. More confirmatory chemical data has now been obtained and problems which arose from the inherent instability of the compound encountered during previous work have been clarified. Purification of the compounds through elaborate chromatography and recrystallization has provided the proper analytical data for the following: (25R)-22,26-imino-5α-cholest-22(N)-en-3β-ol (I), (25R)-22,26-imino-5α-cholest-22(N)-en-3β,16α-diol (II) and 22,26-imino-5α-cholest-22(N)-en-24-on-3β-ol (III). The indefinite melting point of compound II has been resolved. It was found to be due to the presence of a trace of the hydrochloride. Compound III was found particularly unstable to alkali, and one of the degradation products has been identified as 3β-hydroxybisnorallocholanic acid. This augurs well for the assigned structure III.

Significance to Biomedical Research and the Program of the Institute: The isolation of steroidal alkaloids bearing an unsaturated piperidino moiety on C-20 may be of biogenetic interest. The Solanum and Veratrum alkaloids (e.g., tomatidine, solasodine, solanidine, solanocapsine, Veratramine and jervine) could originate from such an intermediate. Moreover, since I is present in the plant in fair yields, it might find utility as starting material for the preparation of biologically active steroid hormones.

Honors and Awards: None

Publication:

Sato, Y.: Steroidal Alkaloids. The Chemistry of the Alkaloids. New York, N. Y., Reinhold Publishing Co. (in press).

Serial No. NIAMD-LC-34
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Microbial Hydroxylation of (25R)-22,26-imino-5α-cholest-22(N)-en-3β-ol (Solacongestidine)

Previous Serial No.: None

Principal Investigator: Hidesato Kataoka

Other Investigator: Yoshio Sato

Cooperating Unit: None

Man Years (computed for the 12 month period)
Total: 2/3
Professional: 2/3
Other: 0

Project Description:

Objective: To convert the alkamine (25R)-22,26-imino-5α-cholest-22(N)-en-3β-ol, isolated from Solanum congestiflorum, to some known steroidal alkaloid in support of our proposed biogenetic pathway. Especially, hydroxylation at the C-16β position was desired to afford dihydrosolasodine, one of the well-known steroidal alkaloids present in the Solanum sp.

Method Employed and Major Findings: Solacongestidine was incubated with five strains of fungi, i.e., Corticium centrifugum, Streptomyces roseochromogenus, Wojnomicia graminis, Mycosphaerella latebrosa and Cephalosporium acremonium, well known for their ability to hydroxylate steroids in the C-16α or β position. Each strain was grown individually in a medium prepared according to the literature. The alkamine was suspended in the broth and shaken aerobically at about 28°C for several days. The product was checked intermittently, but no 16α or β-hydroxylated compound or its cyclized spiroaminoketal derivative (dihydrosolasodine) was obtained.

A product isolated from the broth of Corticium centrifugum is being investigated. Its mass spectrum, IR, and UV data indicate that the compound possesses one hydroxyl and one ketonic carbonyl function, while the piperideine side chain remains intact as judged from the strong m/e 125, 111 peaks and from the spectral data at 240 mμ and 6.05 μ. This hydroxyl is not readily acetylated with acetic anhydride-pyridine reagent, but oxidized easily with Kiliani's reagent to provide a derivative with two six-membered ring ketones. Accordingly, the hydroxyl is thought to be located in the sterically most hindered 11β-position, with the ketone at C-3 where the original β-hydroxyl function was located. The NMR spectra of the fermentation product is in agreement for a steroid possessing the features of a 3-keto-Δ^4-11β-hydroxyl structure. Therefore, the structure (25R)-22, 26-imino-11β-hydroxy-5α-cholest-22(N)-en-3-one is tentatively being proposed for the fermentation product.

Significance to Biomedical Research and the Program of the Institute: Hydroxylation of solacongestidine at C-16β followed by cyclization to soladulcidine (dihydrosolasodine) would lend support to our view that solacongestidine is the biogenetic precursor to a number of steroidal alkaloids of the Solanum and Veratrum species. Hydroxylation at the epimeric C-16α position would afford the alkamine, solafloridine, a new approach to its synthesis and additional proof of its structure. Moreover, the microbial oxidation of solacongestidine could confer activity or produce intermediates of some utility.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-35
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Synthesis of Solacongestidine

Previous Serial No.: None

Principal Investigator: Rosita T. Li

Other Investigator: Yoshio Sato

Cooperating Unit: None

Man Years (computed for the 12 month period)
Total: 1 1/3
Professional: 1
Other: 1/3

Project Description:

Objective: To investigate the synthesis of solacongestidine and related compounds by the reaction of 3β-acetoxy-20α-bromo-5α-pregnane with either α-methyl-δ-chlorovaleronitrile or 5-methyl-2-piperidone.

Methods Employed: 3β-Acetoxy-5α-pregnan-20β-ol was brominated with phosphorus pentabromide in the presence of calcium carbonate to afford the expected 3β-acetoxy-20α-bromo-5α-pregnane. The magnesio derivative of the latter is to be reacted with either α-methyl-δ-chlorovaleronitrile or 5-methyl-2-piperidone to give solacongestidine.

Major Findings: Contrary to expectations, the bromination of 3β-acetoxy-5α-pregnan-20β-ol with phosphorus pentabromide led to D-homoannulation to afford the two bromo derivatives, 3β-acetoxy-17aα-and 17aβ-bromo-17α-methyl-D-homo-5α-androstanes. The 17α-methyl-D-homo structure was established by reduction of the bromo compounds to 17α-methyl-D-homo-5α-androstan-3β-ol and comparison with an authentic sample derived from the lithium aluminum hydride reduction of the known uranediol 3-acetate 17a-tosylate. The assignment of the 17aα and 17aβ configurations for the bromo compounds were based on spectral studies.

These observations led us to reexamine the published work of Adam and Schreiber [Tetrahedron Letters, 923 (1965); Tetrahedron 22, 3581 (1966)] on the chlorination of 3β-acetoxy-5α-pregnan-20β-ol. The reported 3β-acetoxy-20β- and 20α-chloro-5α-pregnanes were in fact proven to be the C-17a epimeric chloro-17α-methyl-D-homo-5α-androstane-3β-acetates.

The chlorination of 3β-acetoxy-5α-pregnan-20α-ol, unlike the 20β-hydroxy compound, gave mainly 3β-acetoxy-20α-chloro-5α-pregnane.

Significance to Biomedical Research and the Program of the Institute: Solacongestidine is a possible precursor of solanidine, solasodine and many other related steroidal alkaloids. The successful synthesis of solacongestidine would allow us to study the biosynthesis of this type of compound by employing a radioactive precursor. Our observations on the rearrangement of 3β-acetoxy-5α-pregnan-20β-ol to the epimeric 17a-halo-17α-methyl-D-homo steroids with some halogenating agents provokes interest and sheds some light on the mechanism of the D-homoannulation rearrangement. Since the epimeric 20α-hydroxy compound gives mainly the substituted 20α-halo derivative, it seems that the heterolysis of the intermediate 20β-ester-halide complex is anchimerically assisted by the migration of the C-16,17 bond to C-20. Further studies on the scope (C_{17}-iso-20α,β-hydroxy and C-20α-hydroxy steroids) of the reaction is being pursued. Alternate methods for the preparation of C-20 halo compounds is also being sought.

Honors and Awards: None

Publications:

Li, R. T. and Sato, Y.: D-Homoannulation of 5α-Pregnane-3β,20β-diol 3-Acetate with Phosphorus Pentabromide. J. Org. Chem. (in press).

Serial No. NIAMD-LC-36
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolism of C^{14}-Labelled Steroids by Adrenals from Rats with a Mammotropic Pituitary Tumor

Previous Serial No.: NIAMD-LC-43

Principal Investigator: David F. Johnson

Other Investigator: Daniel Francois

Cooperating Unit: Robert W. Bates, LNE, NIAMD

Man Years (computed for the 12 month period)
  Total: 1
  Professional: 1
  Other: 0

Project Description:

Objective: Continuation of the study on effects of long-term stress by ACTH, growth hormone, and prolactin on steroid biogenesis in rat adrenals. Also, study of drug effects on biogenesis in these stressed adrenals.

Methods Employed: Radioactive progesterone, deoxycorticosterone or corticosterone was incubated with normal rat adrenals and adrenals from rats subjected to long-term stress of tropic hormones of the anterior pituitary. Extracts of the incubates were chromatographed on silicic acid columns by gradient elution chromatography. Individual fractions were analyzed by plotting U.V. and colorimetric tests versus radioactivity. Isolated zones of radioactivity were further characterized by thin-layer chromatography and radioactive scanning.

Major Findings: In earlier studies we found that *in vitro* conversion of progesterone was 30 times greater by normal adrenal tissue than with the adrenal tissue from tumor animals. Investigation of the progesterone metabolites further showed that steroid biogenesis in the adrenal tissue of tumor animals occurred via a different pathway than in controls. This fact was evidenced by production of corticosterone and 18-hydroxydeoxycorticosterone in normal adrenals and only 18-hydroxycorticosterone in the adrenals of tumor rats. The effects of drugs that inhibit 11β-hydroxylation (Metopirone) and conversion of cholesterol to pregnenolone (Eliptin) on the biosynthetic pathways in these stressed adrenals is at present under study.

Significance to Biomedical Research and the Program of the Institute: As reported previously, these tumor-bearing rats showed lowered blood cholesterol and splanchnomegaly of the liver, kidney, and heart. The fact that splanchnomegaly did not occur in adrenalectomized rats with MtT, and administration of adrenal steroids to adrenalectomized MtT rats did not exhibit marked splanchnomegaly, suggested that some unknown factor of adrenal origin was the possible causative agent. The present use of drugs which inhibit specific steps in the biosynthetic pathways of adrenal steroids may lead to an elucidation of these agents.

Honors and Awards: None

Publication:

Francois, D., Bates, R. W., and Johnson, D. F.: In Vitro Metabolism of 4-^{14}C Labelled Progesterone by Adrenal Glands from Rats with a Transplantable Mammotropic Pituitary Tumor. Endocrinology 81, 246-250, Aug. (1967).

Serial No. NIAMD-LC-37
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Adrenal Metabolism of the Gerbil

Previous Serial No.: NIAMD-LC-44

Principal Investigator: Daniel Francois

Other Investigator: David F. Johnson

Cooperating Unit: H. Y. C. Wong, College of Medicine, Howard University

Man Years (computed for the 12 month period)
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: Continuation of the study of in vitro effects on the adrenal metabolism of this new experimental animal, particularly the effect of the hypocholesterolemic drug triparanol (MER-29).

Methods Employed: Progesterone-4-C^{14} was incubated with gerbil adrenal tissue in vitro, following prolonged treatment of the animal with triparanol. Radioactive metabolites of progesterone were analyzed by column and thin-layer chromatography.

Major Findings: In the continuing effort to find an effective hypocholesterolemic agent, considerable attention was directed toward the mode of action of one such agent, triparanol (MER-29). Following the first demonstration of diminished blood cholesterol in rats by Blohm et. al., other investigators identified the observed effect as an inhibition of the conversion of desmosterol to cholesterol. Wong et. al. further showed that atherogenic effects in the gerbil were less, and the lowering of blood cholesterol occurred only after prolonged treatment as compared to the effects in rats. For this reason, and the more important fact that little attention had been directed to the effects of this drug on adrenal metabolism of progesterone, prompted the present study.

It was found that triparanol treatment resulted in marked inhibition of the 21-hydroxylating enzyme systems of adrenal glands from test animals, reflected in reduced metabolism of radioactive progesterone and diminished production of its normal metabolic products. Little effect was observed on the 11β-hydroxylase enzymes.

Significance to Biomedical Research and the Program of the Institute: The detailed analysis of in vitro effects of the drug triparanol on adrenal steroid metabolism has revealed a direct effect on the transformation of progesterone to important steroid metabolites in addition to the earlier reported effect on cholesterol biosynthesis. The study emphasizes the importance of direct evaluation of the effects, of hypocholesterolemic drugs on the adrenal gland as well as for the production of a decreased cholesterol pool.

Honors and Awards: None

Publication:

Francois, D., Wong, H. Y. C., and Johnson, D. F.: Effects of Triparanol (MER-29) on Progesterone-4-^{14}C Metabolism in the Adrenal Gland of the Mongolian Gerbil. Steroids 10, 115-125, Aug. (1967).

Serial No. NIAMD-LC-38
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Photosensitized Hydration of Cholesterol and Related Steroid Olefins

Previous Serial No.: None

Principal Investigator: James A. Waters

Other Investigator: Bernhard Witkop

Cooperating Unit: None

Man Years (computed for the 12 month period)
Total:
Professional:
Other:

Project Description:

Objective: To find optimal conditions for the photohydration of steroid monoolefins and to study the mechanism of the process.

Methods Employed: (A) Irradiation of cholesterol (I) in t-butyl alcohol, water, and o-xylene (a photosensitizer which serves as an energy transfer agent) was performed with a Hanovia mercury-vapor lamp equipped with a Vycor filter. (B) Cholesterol was irradiated under conditions described above using deuterium oxide in dioxane-o-xylene.

Major Findings: Photohydration experiments of I have afforded two compounds in excellent yield. Preliminary spectral data indicate products are a hydrated cholesterol compound, i.e., a diol, and an oxide with no other functional groups. The latter is resistant to lithium aluminum hydride reduction (vigorous conditions). The photodeuteration experiments gave two compounds analogous to the above. Mass spectral data indicate two deuterium atoms are present in each compound. Further study of these reactions is in progress.

Significance: This study is of interest not only from a mechanistic viewpoint but also because of the recent interest in polyoxygenated steroids such as the ecdysones (insect molting hormones).

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-39
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Photorearrangement and Sodium Borohydride Reduction of 1,4-cholestadien-3-one

Previous Serial No.: None

Principal Investigator: James A. Waters

Other Investigator: Bernhard Witkop

Cooperating Unit: None

Man Years (computed for the 12 month period)
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objective: To study the mechanism of the photorearrangement of 1,4-cholestadiene (I) in the presence of sodium borohydride.

Methods Employed: (A) 1,4-Cholestadien-3-one (I) in *t*-butyl alcohol was irradiated with a 450 watt Hanovia mercury-vapor lamp. (B) Dienone (I) was irradiated in a similar manner in the presence of sodium borohydride. (C) Ground state reductions of I at various borohydride concentrations were studied. Kinetic data was obtained from each of the above experiments.

Major Findings: Irradiation of 1,4-cholestadien-3-one (I) gave 4 phenols, i.e., 1-hydroxy-4-methyl-19-norcholesta-1,3,5(10)-triene (II, 40%), 3-hydroxy-1-methyl-19-norcholesta-1,3,5(10)-triene (III, 40%), 4-hydroxy-2-methyl-19-norcholesta-1,3,5(10)-triene (IV, 10%), and 2-hydroxy-4-methyl-19-norcholesta-1,3,5(10)-triene (V, 4%). Irradiation of I in the presence of sodium borohydride afforded phenol II (15%), phenol III (45%), phenol IV (13%), 4-methyl-19-norcholesta-1,3,5(10)-triene (5%), and an unidentified sterol. Ground state borohydride reduction of I gave 5β-cholest-1-en-3-one (10%), 4-cholesten-3-one (18%), 4-cholesten-3β-ol (40%) and a hydrocarbon.

Significance to Bio-medical Research and the Program of the Institute: The formation of phenols in lieu of sodium borohydride reduced compounds in this irradiation-borohydride reaction is in direct contrast to the photoreduction of 4-cholesten-3-one (ref. below) in which the compounds formed (rate increase factor 22.5) were essentially the same as ground state reduction products. Apparently this is due, in general, to the higher rate and quantum efficiency of triplet rearrangement in dienones in comparison to enones, coupled with the fact that the borohydride reduction of the ground state species is extremely slow.

Honors and Awards: None

Publications:

Waters, J. A. and Witkop, B.: Sodium Borohydride Reduction of Steroidal Ketones with an without Irradiation. J. Am. Chem. Soc. 90: 758-763, Jan. 1968.

ANNUAL REPORT SUMMARY

LABORATORY OF EXPERIMENTAL PATHOLOGY

Introduction

The position of the Chief of the Section on Biophysical Histology, vacated in July 1966 when Dr. Spicer left NIH, was filled in August 1967. Dr. Ned Feder, previously associated with the Biological Laboratories at Harvard University, joined the Laboratory as the Chief of this Section. After his arrival, a long standing internal discussion among the members of the staff was resumed dealing with the need for the creation of a Section on Chemical Pathology. The function of this Section, simply stated, would be to combine biochemical and electron microscopic methods in the study of normal and abnormal events at the ultrastructural level. This approach had been pursued by individual members for some time in collaboration with scientists outside the Laboratory. It was felt, however, that the addition of such a Section to the Laboratory would, in due course of time, greatly strengthen the activities of the Laboratory itself. With the greatly appreciated support of Dr. J. E. Rall, the Scientific Director of our Institute, steps were undertaken during the past year to create such a Section on Chemical Pathology. Dr. Vincent Marchesi presently in the National Cancer Institute accepted the challenge to start the new Section and will join our Laboratory as the first Chief of the Section on Chemical Pathology in June 1968. Space and positions will come from the Section on Pathologic Anatomy which will cease to function as such.

Activities such as the Tissue Preparation Unit and the animal caretakers and animal quarters will be placed under the Office of the Chief of the Laboratory. These as well as the responsibility for diagnostic surgical pathology service conducted in this Laboratory for the Division of Indian Health of the Public Health Service, the Hospital of the Coast Guard Academy in New London, Connecticut, and several of the Federal Prisons will be under the Chief of the Laboratory until the return of Dr. Benjamin Highman from his present assignment as Liaison Officer of the Public Health Service to the Armed Forces Institute of Pathology, who will take over these duties.

As in previous years, the Laboratory expresses its deep appreciation to several pathologists in other units at NIH who unselfishly have helped with their diagnostic skill in surgical pathology. Dr. Ruth Kirschstein (Chief of Pathology, DBS), Dr. Allen W. Cheever (Chief of the Section on Host-Parasite Relations, LPD-NIAID) and Dr. Harry M. Givelber (Biology Branch, NCI) participated at regular intervals throughout the year.

Finally the last year has seen the culmination of the hard work and of the ever active and imaginative mind of Leon Sokoloff, Chief of our Section on Rheumatic Diseases, in the completion of his monograph on "The Biology of Degenerative Joint Disease" in the process of publication by the University of Chicago Press. Those of us who were privileged to read the manuscript have taken great delight in the scholarly and clear descriptions of what is known and of what remains to be done in the future. This is a remarkable book, indeed, and the Laboratory can be proud that it has sprung from the mind and pen of one of its members.

Pathology of Rheumatic Diseases

The Section on Rheumatic Diseases has conducted systematic studies on the biology of degenerative joint disease over the past 15 years employing, at different times, nutritional, biomechanical and certain chemical techniques. It has questioned long held views that osteoarthritis is an inevitable concomitant of aging and necessarily irreversible. The recent development by others of methods for in vitro culture of cartilage cells has shown that adult articular chondrocytes, far from being unable to regenerate, are in fact capable of turning over once they have been freed from their matrix. This approach may open up a new dimension in this area of biology and the Section has started to use it as a principal tool in the next phase of its work.

Studies of the lubrication of animal joints have been brought to a successful conclusion and must be regarded as a major accomplishment of the laboratory. The mechanical performance of dog ankles in vitro has shown that mixed modes of lubrication, primarily hydrostatic and boundary, are involved (Mr. Linn). In addition to refining data concerning the separate roles of the protein and polysaccharide components of synovial mucin in boundary lubrication, it was observed that heparin reduces the effectiveness of this mechanism (Mr. Linn and Dr. Radin). The functional significance of the protein moiety has also been confirmed by Mr. Wilkins who showed that proteases destroy synovial boundary lubrication of a glass-rubber bearing, a test system that eliminates the hydrostatic component of lubrication derived from the cartilage. The lubricating properties of human synovial fluids from a variety of conditions has been studied for the first time. Mr. Linn and Dr. Radin have found this property to be well preserved in two cases of rheumatoid arthritis. Dr. McCutchen has designed and built a micro Couette viscometer for use with the small samples of synovial fluid available for friction measurements.

The pigment of aging cartilage described in last year's report has been partly characterized chemically. Its resistance to exhaustive proteolytic digestion permitted it to be freed from the tissue by pancreatic protease. The pigment is dialyzable and associated with the non-collagenous protein fraction (Drs. van der Korst, Sokoloff and Miller).

Spinal ankylosis was observed in two of five Macaca monkeys between 16 and 35 years of age. One of the lesions closely resembled Marie-Strümpell disease, a condition not known to occur in species other than man. The other was of a type reported in several species. The interpretation is offered that it corresponds to the recently recognized human disorder, hyperostotic spondylosis (Drs. Sokoloff, Snell and Stewart).

Articular cartilage is attached to bone through a zone of calcification and the precise mode of this attachment would seem to be an important physical property of joint tissue. Microradiographic studies (carried out by Drs. Green and Sokoloff in collaboration with Dr. E. D. Eanes and Mr. G. Martin, NIDR) disclose that the calcified layer in man is more densely mineralized than the subchondral bone. Variations in this interface with age, species and disease as well as experimentally induced remodeling are under investigation.

Studies in Cytogenetics

Cytogenetic investigations on the effect of LSD: Interest in this work was stimulated by conflicting reports of the adverse effects of LSD on the chromosomes of human leukocytes *in vitro* and *in vivo*, and on the embryos of rats, mice and hamsters. A pilot *in vitro* experiment with human blood cultures was performed with LSD in the final concentrations: 10 mg/cc, 1 mg/cc, 0.1 mg/cc, 0.01 mg/cc, and 0.001 mg/cc were added to the cultures at 0, 6, 24, and 68 hours after starting the cultures. We found up to ± 80% of the metaphases with chromosomal alterations in all the cultures that received LSD at 0 hour, independent of the dosage. However, in all the cultures receiving LSD at 6, 24, and 68 hours, this frequency drops to ± 15% while the controls had only ± 10%. The cells are apparently most sensitive to the effects of LSD long before the S period of the mitotic cycle. (At 6 hours the protein synthesis is more or less over, at 24 hours the DNA synthesis period is starting and at 68 hours the DNA synthesis is almost finished.) Several studies are contemplated to confirm and extend these observations including cytogenetic studies on the chromosomes of leukocyte cultures from the Spring Grove State Hospital from a series of patients before, during and after therapy. Thus far, we have studied about 100 blood samples of which only 5 have shown considerable chromosome damage (Dr. Tjio).

Cytogenetic studies on a mouse translocation: Cytogentic studies were carried out on a mouse translocation with reduction of chromosome number but constant number of arms. The animals were accidently found in a colony of albino mice which had been randomly bred at the NICHD for over three years without any known mutagens. It appeared to be the result of centric fusion of the smallest autosome and one of the larger autosomes of the complement. Chromosome number of heterozygotes was 39 and that of homozygotes 38. The animals with 38 and 39 chromosomes were phenotypically normal with the exception of one female runt found in a litter from a mating between homozygotes. Studies of meiosis of male heterozygotes showed an autosomal trivalent at metaphase I and a low incidence of unbalanced metaphase II cells. The homologous translocation submetacentrics formed a ring bivalent at diakinesis in male homozygotes, and metaphase II cells of these animals were balanced. Progeny from matings between heterozygotes had balanced complements (N.F. = 40) of 38, 39, or 40 chromosomes in a near --1:2:1 ratio. The homozygotes and their non-translocation-bearing littermates demonstrated normal fertility, and fertility of heterozygotes was moderately reduced (Drs. White and Tjio).

Studies on Transfer Ribonucleic Acid Synthesis

A method for the study of tRNA synthesis has been developed which includes pulse labeling tRNA and fractionating it on a column. Radioactive guanine is used, and there is evidence that guanine incorporation reflects the DNA dependent portion of tRNA synthesis. The synthesis of a new tRNA species accounting for 2% or more of the tRNA synthesized during a period of labeling can be detected.

tRNA synthesis was studied during the first five minutes following induction of the lactose operon in *E. coli* K 12. Two possibilities were of interest in these experiments. One was that tRNA played a central role in enzyme induction, and that the synthesis of the enzymes of the operon was controlled by the presence of specific tRNA species. The other was that synthesis of certain tRNA species might be induced or inhibited concurrently with alterations in protein synthesis such as occur with induction of the lactose operon. No difference was found, however, between tRNA synthesis in induced and control cells in the experiments (Dr. Smith).

tRNA synthesis has also been investigated during the initial five minutes of T2 bacteriophage infection in *E. coli*. The pattern of tRNA synthesis in infected cells was markedly different from that in control cells (Drs. Tillack and Smith).

Protein Components of Animal Cell Membranes: Isolation and Characterization

Membrane fractions can be prepared from red blood cell ghosts which are essentially free of erythrocyte cytoplasm or serum proteins, and these have been used as a source of membranes for studies of chemical composition. Approximately one-half of the dry mass of animal cell membranes consists of protein, and very little is known about its chemical composition and physical properties. A protein can be solubilized from the membranes of erythrocytes of several species (human, guinea pig, horse, rabbit, sheep, and rat) which makes up at least 20% of the membrane protein and is quite homogeneous in all species examined. This protein is extracted from the red cell membranes of these species by treatment of the membranes with low ionic strength media containing chelating agents and sulfhydryl compounds. The soluble protein obtained from all the species of red cells appears to be identical by several critera: polyacrylamide gel electrophoresis; analytical ultracentrifugation studies; amino acid analyses, and immunoprecipitin reactions. We are currently completing this comparison of the chemical properties of the soluble proteins derived from erythrocytes of different species, and experiments are under way to devise methods of extracting and characterizing the remaining proteins of the red cell membrane (Drs. Tillack, Marchesi [GL&C-NCI] and Steers [LCB-NIAMD]).

Autoradiographic and Cytochemical Studies Regarding Structure and Function of Nucleolus.

The synthetic functions of the nucleolus in the early stages of Rous Sarcoma virus infection have been investigated. The cellular pathology underlying the "neoplastic" transformation of Rous Sarcoma virus infected cells remains poorly understood and compared with that of the small RNA viruses little is known about the site and manner of intracellular viral RNA synthesis. Studies with metabolic inhibitors of RNA synthesis, such as Actinomycin, and inhibitors of DNA and protein synthesis, have shown a complex interdependence of infectivity and transformation with cellular DNA and protein synthesis varying with the time of administration of the inhibitors; but have failed to clarify convincingly the mechanism or the site of viral RNA synthesis. Conflicting reports concerning inhibition of intracellular viral RNA by Actinomycin may be due in part to the complexity of physical separation of a small amount of rapidly degraded viral RNA in a large excess of host RNA. Using quantitative autoradiographic techniques experimental data has been obtained which support the interpretation that the nucleolus is a site of synthesis or assembly of viral RNA.

Secondary cultures of RIF free chicken fibroblasts were protected for 3 hours with 0.1 or 0.2 μgm/ml of Actinomycin, and were washed and infected with Bryan strain RSV in a multiplicity of about 100. Control cells were either mock-infected in the suspending medium or exposed to a heat-inactivated virus suspension. At varying times following infection the cells were then pulse-labeled with tritiated uridine for 10 minutes so that the label appeared only in the nucleus. Differential grain counts of the nucleolus and nucleus were made in radioautographs prepared as previously reported, using groups of 25 randomly selected cells.

It was found that nucleolar RNA synthesis is transiently increased after Actinomycin inhibition in cells subsequently infected when compared with infected cells not previously inhibited and with respect to uninfected control cells. This relative increase of nucleolar RNA synthesis occurs around 10 hours post infection, disappearing by 24 hours and precedes an equivalent rise in infected cells not previously inhibited. These effects are not observed with the heat-inactivated virus inoculum. The labeling of ribosomal protein in the nucleolus, using a short pulse of tritiated lysine, shows no such increase after virus infection and is identically inhibited by Actinomycin in infected and uninfected cells. During the time that nucleolar RNA synthesis is increased in infected cells DNA synthesis is identically inhibited in infected and uninfected cells. However, shortly before the appearance of morphologic transformation the rate of DNA synthesis is faster in infected control cells than in Actinomycin-treated infected or uninfected cells.

The findings that increased synthesis of nucleolar RNA in RSV-infected cells occur under conditions in which nucleolar ribosome synthesis is selectively inhibited support the interpretation that the nucleolus is a site of synthesis or assembly of a viral RNA not inhibited by Actinomycin. The dissociation of this nucleolar RNA label from nucleolar ribosomal protein also strongly suggests that this label is a viral RNA separate from host nucleolar ribosomes. It is proposed that under these experimental conditions a viral RNA may compete with Actinomycin for some G-C rich template sites of ribosomal precursor RNA in the nucleolus. However, the possibility that the nucleolar protein inhibited by Actinomycin may act as an interferon-like agent, inhibition of which could accelerate nucleolar RNA synthesis in infected cells is under experimental consideration (Dr. Suskind).

Studies on Amyloidosis

Studies on the components of human amyloid deposits have indicated that there are two morphologically characteristic components, a periodic rod which has been shown to be composed of unit structures and a nonperiodic fibrillar structure which has shown to be the major component of amyloid deposits. A comparison between the physical and chemical characteristics of these two structures has been facilitated by a purification procedure which eliminates contamination of one component by elements of the other, and the following evidence that these structures are not interconvertable has been obtained. Amino acid analysis indicates that the periodic rod is deficient in tryptophan and methionine while the fiber contains both these amino acids. Both structures have a low uronic acid composition but the periodic rod has 5% neutral sugars while the fiber 2%. Peptide mapping has shown that a tryptic digest of these two structures produces no peptides in common. After denaturation in urea or guanidine, on disc electrophoresis no protein bands are found to be common to both components. X-ray diffraction reveals an amorphous pattern for the periodic rod while the fibril has a consistent and well defined pattern showing a 4.75 Å band indicating it to be a fibrous protein of keratin type. In addition staining reactions of the two components reveal only the fibril to have Congo red birefringence under crossed polars while immunologic studies indicate that only the periodic component is antigenic. It is concluded that the periodic rod is an antigenic globular glycoprotein which does not contribute to the formation of the nonantigenic amyloid fibril, a structural glycoprotein of keratin type.

Immunochemical studies were carried out with human amyloidotic spleen and liver in attempts to obtain a better understanding of the nature of amyloid. Rabbit antisera obtained by prolonged immunization, after suitable absorption with normal tissue antigens, did not react with amyloid preparations purified by fractional centrifugation or proteolytic degradation. They reacted with a protein present in serum and in amyloid deposits, possibly by absorption from the former. These studies suggest that amyloid like the other members of the fibrous protein family is either an extremely poor antigen or nonimmunogenic.

Lack of a satisfactory method for inducing amyloidosis in laboratory animals has been a major factor in our poor understanding of the pathogenesis of amyloidosis. Studies directed at evolving a simple procedure for experimental amyloidosis revealed that amyloidosis developed in mice two weeks after the second of two biweekly intraperitoneal injections of 0.25 ml of an emulsion made up of 25 mg casein, 1.5 ml complete Freund's adjuvant (F.A.) and 1.5 ml buffered saline (PBS). Incorporation of additional M. butyricum, without casein in F.A. reduced the time required for the induction of amyloid. A single intraperitoneal injection of 0.25 of an emulsion of 25 mg M. butyricum, 1 ml. PBS and 1 ml complete F.A. resulted in amyloidosis in mice in two weeks. This procedure for experimental amyloidosis involving a single injection facilitated studies on the sequential cellular changes associated with the formation of amyloid, and attempts at modification of the disease with immunosuppressive procedures, such as the administration of cyclophosphamide (CPP) (Drs. Glenner, Ram and DeLellis).

Carotid Body: Mode of Chemoreception

Surgically excised human carotid bodies have been analyzed by electron microscopy. Several ultrastructural features suggest that they are closely related to sympathetic neuroendocrine glands and autonomic ganglia: the chief cells synthesize intensely osmiophilic cytoplasmic granules, occasionally develop neuroid processes, and are intimately associated with neurilemmal sustentacular cells. Other lines of evidence indicate that the chief cell secretion is norepinephrine or a closely related bioamine-neurotransmitter. The role of chief cells in mediating chemosensation may be explained by modulated release of the bioamine in response to local metabolic conditions. Human chief cells are richly innervated. Recognizable zones of synapse display an efferent type of polarity and sometimes appear adrenergic. Thus, reflexive sympathetic excitation of chief cells may be physiologically significant in man. In terms of impulse generation, small cell units could create a miniature internal circuit for modulation of simultaneous metabolic and sympathetic efferent stimulus input. Consideration of chief cells as active couples or transducers interposed between nerve endings and the interstitial environment in interpretation of physiologic data is suggested to replace older concepts based on a passive "sensory" mechanism (Drs. Grimley [PA-NCI] and Glenner).

Immunochemical and Immunohistochemical Studies on Prolactin

Immunochemical studies with prolactin were concerned with the localization of prolactin in pituitary cells of rostral pars distalis growing in culture. In these studies fluorescent antibody to ovine prolactin was used to determine the presence of prolactin in pituitary cells of Fundulus. A second project involved the isolation of crude prolactins from the carp and the pollack, the development of antisera to these prolactins and a study of the cross-species precipitin reactions with ovine and bovine prolactins. The series of pollack pituitary extracts resulted in one preparation with a five-fold increase in precipitin reaction per microgram of fish prolactin. A third project was completed on the purification of pollack pituitary extracts by immunochemical binding with antibody and subsequent isolation of the antibody from the antigen by Sephadex column fractionation (Dr. Emmart).

Pathways of Water Transport Across Renal Collecting Tubules.

In a previous study it was shown that interspaces between cells, in the isolated perfused renal collecting tubule, dilate during vasopressin-induced osmotic water flow. This suggested that the interspaces may have a role as part or all of the pathway for water flow across the tubule. The present study has further elucidated the role of the lateral interspaces in bulk water flow and has established beyond reasonable doubt that water does enter the space across the lateral cell membranes and exits via the basal slit or pore between the cells. Oubaine added to transporting tubules stops the short circuit current but does not affect the dilatation of the space indicating that salt is not transported into the space. Swelling is also not due to trapped solute because the spaces do not dilate when tubules are swollen in a hypotonic medium. The placing of control or swollen tubules in a hypertonic or isotonic medium produces large intercellular spaces. This could occur only if the basal slit were patent to water and solute and water entered into the space across the lateral cell membranes, since the basement membrane is not a rigid structure. Further evidence of the patency of the pore is provided by separation of the cell membranes and the entrance of electron microscopic tracers into the intercellular space even though the tracers do not penetrate into the region of the tight apical junction. The rate of emptying of the intercellular spaces was shown to be directly dependent on the intraluminal hydrostatic pressure which in turn depended on the elastic properties of the basement membrane. Alternatively with the shrinkage of cells to a point where the basement membrane offered no positive pressure the dilated spaces did not collapse with time. Evidence that water flows across the basal cell membrane as well as across the lateral cell membranes was obtained by observations that the shrinkage of tubules swollen to different degrees was always biphasic with an initial rapid rate due to movement of water across the basal cell membrane and a long slower expodential rate due to emptying of the intercellular spaces. Preliminary calculations suggest that the majority of water flows across the basal cell membrane but that a significant portion also flows through the intercellular space (Dr. Ganote and Dr. J. Grantham [LKEM-NHI]).

Effects of Fixation on Hepatic Ultrastructure

A study was conducted of the effects of different fixation techniques on liver ultrastructure. It was found that a wide variety of hepatic changes could be induced by the fixation techniques now in general use. These artifacts of liver fixation were often similar to those changes reported in the literature as nonspecific normal or pathological lesions. They included a variety of mitochondrial changes, dilatation and vesiculation of the endoplasmic reticulum, clumping of nuclear chromatin and variations of cellular density with formation of light and dark cells. This study stressed the need for rapid and complete fixation of liver cells in order to preserve hepatic ultrastructural details. An improved method of liver fixation was developed using portal vein perfusion at high flow rates (~70 ml/min/100 gm body weight) with a nearly isotonic glutaraldehyde solution. This technique has been used with consistently good results in several ultrastructural studies (Drs. Ganote and Moses).

Studies on the Mechanism of ACTH Stimulation of Adrenal Steroidogenesis

Studies on the mechanism of ACTH stimulation of adrenal steroidogenesis have been continued during the past year. Previous studies had shown that routine procedures for processing tissue for electron microscopy resulted in extraction of over two-thirds of the tritium-labeled cholesterol from rat adrenal cortex. A method of processing was developed which allowed retention of up to 90 percent of H^3-cholesterol and permitted autoradiographic studies at the electron microscope level of corticosteroid-precursor cholesterol pools in rat adrenal cortical cells. Approximately 70 percent of developed grain was found to be over the lipid droplets in the hypophysectomized control animals. With ACTH stimulation, there was depletion of counts from all cell fractions, but the bulk of the depleted grain was from the lipid droplet fraction. This depletion was blocked by cycloheximide which caused a slight increase in specific activity in the non-lipid droplet, non-mitochondrial cytoplasm.

Biochemical studies were also carried out for correlation with the electron microscope autoradiographic data. A method of obtaining purified fractions of adrenal cortical cell lipid droplets was devised. Determinations of the distribution of relative counts in free and esterified cholesterol in the purified lipid droplet fraction and in cell fractions obtained by routine procedures of differential centrifugation were in general agreement with the relative distribution of counts as determined by electron microscope autoradiography. Corticosterone production and specific activity of tritium label was determined on the various experimental groups in vivo and in prelabeled adrenal slices incubated in vitro in the presence and absence of ACTH and/or cycloheximide.

The data obtained were consistent with the following interpretations: (1) Exogenously administered cholesterol was incorporated into the adrenal cortical cell lipid droplet in esterified form. (2) The lipid droplet cholesterol probably represented a major pool of steroid precursor cholesterol. (3) ACTH stimulation caused conversion of esterified to free cholesterol which was transported into mitochondria and converted to pregnenolone and through subsequent steps to corticosterone. (5) Cycloheximide blocked the cholesterol-to-pregnenolone step and resulted in accumulation of the metabolic intermediate (free cholesterol) in the soluble cell fraction as determined by ultracentrifugation or in the non-lipid droplet, non-mitochondrial cytoplasm as determined by autoradiography. It could not be determined, however, whether cycloheximide blocked transport of free cholesterol into the mitochondria or inhibited a metabolic step within mitochondria with rapid back-diffusion of the unmetabolized cholesterol (Dr. Moses, Dr. Davis [Hershey Medical Center, Hershey, Pa.], Rosenthal [LKEM-NHI], and Dr. Garren [Yale University, New Haven, Conn.]).

Ultrastructural Studies of Continuous Cell Lines Derived from Peripheral Blood of Patients with Infectious Mononucleosis.

Continuous suspension cultures derived from peripheral blood leukocytes of patients with infectious mononucleosis have been established (Drs. Glade and Chessin [LCI-NIAID]). Under identical conditions, peripheral blood leukocytes from control individuals did not proliferate in long-term culture. Electron microscopic studies of fourteen cell lines derived from infectious mononucleosis patients has been carried out. These cells in culture had a lymphoblastoid appearance. The herpes-like virus (HLV) was identified in four of the fourteen cell lines examined. The HLV has been identified by other investigators in lymphoid and myeloid cell lines derived from patients with a variety of disorders, including Burkitt's lymphoma, leukemia, a variety of malignancies, and apparently healthy individuals. A far more consistent structural finding in the infectious mononucleosis cell lines was the presence of reticular aggregates of 22 mμ particles present in association with the rough endoplasmic reticulum. These dense aggregates were found in a relatively high percentage of cells in all of the fourteen cell lines examined. Similar structures have been described previously in meningioma and meningeal sarcoma cells induced in dogs by Rous sarcoma virus, in sarcoma cells induced in monkeys by the Rous virus, in cell lines derived from Burkitt's lymphoma and in cultured monkey kidney cells infected with rubella virus. Also present in the infectious mononucleosis cell lines were small (25-50 mμ) extracellular virus-like particles which were morphologically distinct from the HLV. These small extracellular particles were more numerous in those four cell lines which contained both the HLV particles and the cytoplasmic reticulum of 22 mμ particles. Attempts to transmit infective particles from these cell lines, however, have so far been unsuccessful. Studies are currently under way to determine if the detected virus-associated or virus-like particles are indeed related to a transmissible agent which may be involved in the pathogenesis of infectiou mononucleosis and the ability of peripheral blood leukocytes to survive in long-term culture (Dr. Moses and Drs. Chessin, Glade and Kasel [LCI-NIAID]).

Histochemical Studies: Methods and Applications

Adenosinetriphosphatase: There is skepticism about the validity of current methods for the histochemical demonstration of ATPase, in large part because of uncertainty about the inhibiting effects of lead. It was found that the stability constant of lead-ATP complexes is of the same order of magnitude as that of ATP complexes with calcium and magnesium. Thus Pb competes with Mg for ATP when both cations are present. When ATP is present in excess, free Pb is significantly reduced. Precipitation of lead phosphate is affected by the quantity of ATP present in the medium as well as by Ca or Mg added as activating cations. At the same time, significant quantities of nucleotide substrate may be lost to precipitate.

With two microsomal ATPases (liver and kidney) enzyme inhibition by lead can be largely overcome by increasing ATP concentrations--whether by reversing the effects of competitive inhibition or by chelation effects, or both, is not clear.

The results of these biochemical studies of ATPase have a direct bearing on interpreting histochemically demonstrated ATPase. The results can also be applied to the development of improved methods for the histochemical demonstration of this enzyme (Dr. Tice).

Hydrophilic embedding media for lipid and enzyme histochemistry: Work on the development and use of hydrophilic embedding media is in progress. With some of these media, one can under suitable conditions preserve and demonstrate lipids and enzymes that ordinarily are lost or destroyed (Feder, *J. Cell Biol.* 19: 23A, 1963); at the same time the preservation of tissue and cell structure is excellent as revealed by light and electron microscopy. A report on the use of glycol methacrylate as an embedding medium has appeared recently (Feder and O'Brien, *Amer. J. Botany* 55: 123-142, 1968); studies on the tissues of humans and laboratory animals are under way (Dr. Feder).

"Microperoxidases" as Histochemical Markers

Certain heme-peptides of relatively low molecular weight (900 to 2000) have peroxidatic activity sufficiently high so that they can be demonstrated histochemically at the level of the light and electron microscope. These "microperoxidases", because of their small size, have advantages as a histochemical marker over horseradish peroxidase (molecular weight about 40,000), which has been used as a marker for antibodies (Nakane and Pierce, *J. Cell Biol.* 33: 307-318, 1967) and as a means of delineating channels of tissue fluid movement (Karnovsky, *J. Cell Biol.* 35: 213-236, 1967) (Dr. Feder).

Bacterial Periplasmic Space as a Functioning Entity

Certain enzymes (cyclic phosphodiesterase, glucose-6-phosphatase, and alkaline phosphatase) have been demonstrated in the periplasmic space of E. coli, especially in polar enlargements of this space. These observations are reinforced by analysis of a "minicell" strain of E. coli, which buds off terminal, anucleate cell fragments with dilated periplasmic spaces corresponding to the polar enlargements; these spaces are cytochemically reactive for the enzymes studies. These enzymes can also be shown biochemically to exist in disproportionately high concentrations in the buds. Instead of shrinkage artifact, the periplasmic space can now be regarded as a site where certain enzymes are compartmentalized (Dr. Wetzel).

Thyroid Studies

Accompanying the predominant type of follicle in the thyroid gland of C_3H mice is a system of unusual follicles, whose fine structure was examined. The second system of follicles contains typical follicular epithelium and parafollicular cells, but is characterized by the presence of 1) heterogeneous colloid, 2) occasional ciliated epithelial cells, and 3) a hitherto undescribed and unusual cell type distinguished by abundant agranular reticulum and sometimes by glycogen and secretory globules of unknown nature. The second type of follicle is of interest as a possible derivative of the lateral or ultimobranchial tissue in the embryo (Dr. Wetzel and Dr. Wollman [LPHY-NCI]).

A histological study of thyroid glands from long-term parathyroidectomized rats is being readied for publication. Hyperplasia of thyroid parafollicular cells parallels thyrocalcitonin accumulation; these changes are blocked by parathyroid implants. The parafollicular cell thus appears to be the site of synthesis and storage of thyrocalcitonin (Dr. Wetzel).

Cytodifferentiation in the Maxillary Galea

The maxillary galea of certain saturniid moths is a particularly favorable organ for the study of development because of its highly specialized synthetic activity, abrupt and predictable start of specialized synthesis, and ease of study at all developmental stages. A report on the development of this organ has been submitted for publication (Dr. Feder and Dr. Kafatos [Harvard University, Cambridge, Mass.]).

Cesium Polycarboxylates for Density Gradient Ultracentrifugation

Studies on buoyant media for the ultracentrifugal separation and analysis of nucleoproteins are nearing completion. The value of certain cesium polycarboxylates for ultracentrifugal analysis has been demonstrated. A mechanism has been proposed to explain a) the low buoyant density of nucleic acids and nucleoproteins in concentrated solutions of cesium polycarboxylates, and b) the superiority of these compounds to cesium chloride in their denaturant effects on the ribosomes of higher animals (Dr. Feder and Dr. Kafatos [Harvard University, Cambridge, Mass.]).

Studies on Radiation Pathology

Unshielded rats exposed to 800 R whole-body X-irradiation showed a moderate rise in plasma glutamic oxalacetic transaminase (PGOT) at 6 hours after irradiation and a marked fall in plasma alkaline phosphatase (PAkP) 4 days after irradiation. Shielding the abdomen prevented or greatly lessened these changes. Irradiating only the abdomen produced a relatively small rise in PGOT at 6 hours but caused a marked fall in PAkP at 4 days after irradiation. It is suggested that the rise in PGOT is derived from damaged lymphoid and other radiosensitive tissues and in part from a widespread initial increase in cellular permeability induced by X-irradiation. The fall in PAkP is attributed to radiation injury to the intestinal mucosa (Dr. Highman).

Studies on High Altitude Hypoxia

Continuous exposure of rats to simulated altitude (25,000 ft.) produced uncoupling of mitochondria in 1 day and impaired phosphorylation for as long as 3 days. Four-hour daily exposure to altitude (21,500 ft.) combined with exercise for 1 to 3 days induced elevations in oxidative enzymes in the serum and, in addition, increased the concentration of glycolytic enzymes. Physical training at ground levels prior to the altitude exposures reduced the severity of the early enzyme and tissue changes and increased reproductive performance and survival (Dr. Highman and Dr. Altland [LPB-NIAMD]).

Cycasin Research

Studies concerned with the toxicology and carcinogenicity of the natural glucoside cycasin and of its aglycone part have continued. Various projects which were described in last year's summary report have been published or are presently in press. A general review of the present status of cycasin research was prepared for an UICC sponsored symposium on plant carcinogens.

This report contains a description of four projects which made up the greater part of this particular activity and which have reached or are approaching publication.

Enzymatic hydrolysis of cycasin in the subcutis of neonatal rats: The study originated in an observation that a single injection of cycasin under the skin of newborn rats would result in tumors of kidney, intestines, liver, lung or brain in 85 percent of the animals. Since previous work by others and ourselves had indicated that parenteral or intravenous injections of cycasin were followed by its quantitative excretion in the urine in rats at or beyond weaning age, a special situation appeared to prevail in newborn rats.

Preliminary acute experiments indicated that on a mg/g body weight basis all rats died which had received cycasin subcutaneously between days 1 and 17 of postnatal life, that a few rats survived this treatment between the 17th and 23rd day and that all rats survived when 25 days old or older. The cause of death in these rats was severe centrilobular liver cell necrosis indicating that some hydrolysis of cycasin to its aglycone occurred in rats younger than 3 weeks. Conversely, rats older than 3 weeks had normal livers when subjected to comparable treatment indicating that conversion of cycasin to its toxic aglycone part had not occurred. These results suggested the neonatal period as the optimal time for further experimentation (Dr. Laqueur and Dr. Hirono [Gifu University, Gifu City, Japan]).

The role which the mother rat might play in the hydrolysis of cycasin was explored next. This was indicated since several mothers, themselves untreated but nursing young which had received cycasin subcutaneously, died within 3-4 days and their autopsy findings included severe hemorrhagic liver cell necrosis. The assumption was made that the mother rats while cleansing their young had ingested sufficient amounts of cycasin, hydrolyzed it in the intestinal tract from where the aglycone was absorbed, thus reaching the maternal liver. Simultaneously a part of the aglycone might be excreted with the milk affecting the young. Indeed, each one of these steps was not only possible but had been shown to occur in unrelated experiments. To test the importance of the maternal role more critically, two separate studies were undertaken consisting of a) removal of the injected young from the mother and substituting nursing with artificial feeding and b) repeating the experiment under germfree conditions. In both experiments the young died exhibiting identical changes in the liver. These findings indicated that hydrolysis of cycasin occurred in the young themselves in addition to an unknown percentage of cycasin hydrolysis provided by the mother (Dr. Laqueur and Dr. Hirono [Gifu University, Gifu City, Japan]).

Although information in the literature indicated that the small intestinal mucosa of conventional and germfree rats contained enzymes capable of hydrolyzing synthetic β-glucosides, our observations in germfree rats over the past 5 years had shown that the natural β-glucoside cycasin was not hydrolyzed in the intestine in vivo. Recent studies have produced evidence for the presence and activity of an enzyme in the subcutaneous tissue of prenatal and postnatal rats capable of hydrolyzing not only synthetic β-glucosides but also cycasin. Enzymatic activity was highest in the prenatal and early postnatal period. Activity declined after the 4th day of life and was no longer demonstrable in 25 day-old rats. There was no difference in enzymatic activity when the assays were repeated in germfree rats. While one site for enzymatic hydrolysis of cycasin in newborn rats has thus been clarified affording an explanation for the oncogenic effect previously observed in such animals after subcutaneous injection of cycasin, further studies are now necessary to characterize the enzyme and to look further into its physiologic role during the development of the young rat (Dr. Spatz).

The development of a model system for chemically induced microencephaly: In the course of studies dealing with transplacental induction of tumors in rats, 4 litters were found which had variable degrees of microencephaly at autopsy at the age of 14 months. The degree in reduction of brain weight varied between the litters but was strikingly uniform among the mates of each litter. There was no indication that the brains were malformed and all landmarks were readily identifiable on gross examination. The low weights were essentially due to a marked decrease in the size of the cerebral hemispheres thus exposing broadly the corpora quadrigemina to view. The experimental protocols indicated that the four litters came from mothers which had received intraperitoneal injections of the aglycone on days 14, 15 and 16 of pregnancy, the total amount calculated on the basis of 20 mg of the compound per kg of body weight. Subsequent studies have shown that this type of microencephaly was readily reproducible and data was obtained to examine the postnatal growth of the brain in microencephalic rats in comparison with controls. At all times during postnatal life, the mean brain weights of microencephalic rats consistently were significantly below those of the controls. When such data was plotted on graph paper, the increases in brain weight were comparable in both groups for the first 14 days of life. However, whereas the brains of the controls showed a further increase over a period of one year averaging 510 mg, the respective gain in brain weight in the microencephalics was only 80 mg over the same period. The fairly abrupt stoppage of brain growth at about day 14 suggests that an important step in development of the brain had been prevented from normally proceeding. One significant part of postnatal growth is concerned with myelination. Examination of cross sections of the brain show distinctly less white matter. Whether this is a reflection of a primary neuronal damage resulting in a reduction of axous to become myelinated or whether it is due to an induced defect in myelin formation remains to be determined. Such studies have been initiated.

Additional experiments have shown that the successful production of microencephaly was not strain-limited. Matings of microencephalic sisters and brothers uniformly produced offsprings with normal-sized brains indicating clearly a chemical rather than a genetic effect. It has been found possible furthermore to produce microencephaly with a single dose on either day 14 or day 15 but not on days 13 or 16 of fetal development, and the natural aglycone prepared from cycasin and the synthetic aglycone acetate were equally effective in producing microencephaly (Drs. Spatz and Laqueur).

Collaborative studies were initiated to define a) with histopathologic means the early and late lesions in the central nervous system (Dr. Valsamis, Brooklyn State Hospital, Brooklyn, N.Y.) and b) any deficit in intelligence and sensory functions which might have been initiated in such microencephalic animals (Dr. Haddad, N.J. Bureau of Research in Neurology & Psychiatry, Hopewell, N.J.). Preliminary observations indicate that these explorations have been successful in certain respects. The studies are continuing (with Drs. Valsamis and Haddad).

Specificity of ultrastructural changes in liver cells of rats during acute intoxication: Following the development of fixation procedure of the liver by perfusion techniques which assures optimal fixation by minimizing artifacts (Drs. Ganote and Moses), a comparative study of the effects of several hepatotoxic agents known to produce distinguishable biochemical lesions was undertaken. Two methylating agents, dimethylnitrosamine and methylazoxymethanol (the aglycone of cycasin or MAM), carbon tetrachloride and hydrazine sulfate were used. Under the standardized conditions it was possible to clearly distinguish the very early effects of the two alkylating agents from those of the others. The two alkylating agents produced, however, very similar effects among which nucleolar changes were observable within a few hours. These consisted of an early segregation of the nucleolar granular and fibrillary components with ultimate loss of the granular component. Associated alterations consisted of hypertrophy of the smooth endoplasmic reticulum, formation of membrane whorls, decreases in the number of ribosomes and endothelial damage. Details of the result in this significant study have been submitted for publication (Drs. Ganote and Rosenthal).

Oncogenic effects of MAM in hamsters: Previous experiments in rats in which intraperitoneal injection of MAM had produced carcinomas in the lower duodenum, jejunum and ileum but not in the proximal duodenum or stomach suggested the possibility that the carcinogenic agent might have been excreted with the bile. Since rats do not possess a gallbladder from which to collect sufficient quantities of bile for chemical analysis or in which to study histologically local responses to the carcinogen, hamsters which have gallbladder were used. MAM was given either intravenously as a single dose or intraperitoneally in three divided doses. Among various tumors observed in long-term survivors, two had invasive carcinomas of the gallbladder after intraperitoneal injections of MAM. Studies are now in progress to develop methods for determining MAM and its metabolites in the bile (Dr. Spatz).

Serial No. NIAMD-LEP-1
1. Experimental Pathology
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolic and carcinogenic effects of Cycas circinalis, its glycoside cycasin and its aglycone, methylazoxymethanol in conventional and germfree rats.

Previous Serial Number: Same

Principal Investigator: Gert L. Laqueur

Other Investigators: Dr. Maria Spatz, Dr. C. E. Ganote, Mr. E. G. McDaniel, Dr. H. Matsumoto, Dr. M. Valsamis, Dr. R. K. Haddad.

Cooperating Units: LEP-NIAMD (Drs. Spatz and Ganote)
LNE-NIAMD (Mr. McDaniel)
University of Hawaii (Dr. Matsumoto)
Brooklyn State Hospital (Dr. Valsamis)
N.J. Bureau of Research in Neurology & Psychiatry (Dr. Haddad)

Man Years (computed for the 12 month period)
Total: 6
Professional: 1
Other: 5

Project Description:

The major projects described last year were completed and prepared for publication (see below). One of the major new studies dealing with one of the many biologic effects of methylazoxymethanol, the aglycone of cycasin has been the development of a model for chemically induced microencephaly in rats. The condition is readily reproducible, not strain limited, consistent with longevity, normal attainment of puberty and fecundity. There is no associated microsplanchnia. Genetic changes apparently are not involved, and matings of microencephalic sisters and brothers in an inbred strain of rats uniformly produced offsprings with normal-sized brains. Although the compound had been administered in three divided doses over a three-day period in the earlier studies, it was recently found that the total dose could be administered to pregnant rats on either the 14th or 15th day without producing ill effects or leading to abortion or resorption of the fetuses. A most remarkable feature was the development of brain tumors in 10 percent of the microencephalic rats surviving longer than one year (with Spatz).

Collaborative studies are under way to establish if possible associated pathologic processes which might explain the reduction in size of the brain which essentially involves the cerebral hemispheres (with Spatz and Valsamis). A preliminary study to be presently extended has shown definite intelligence deficit in microencephalic rats which appears to involve their learning ability (with Spatz and Haddad).

Publications:

Spatz, M., Smith, D.W.E., McDaniel, E. C., and Laqueur, G. L.: Role of intestinal microorganisms in determining cycasin toxicity. Proc. Soc. Exp. Biol. Med. 124: 691-697, Mar. 1967.

Laqueur, G. L., McDaniel, E. G., and Matsumoto, H.: Tumor induction in germfree rats with methylazoxymethanol (MAM) and synthetic MAM acetate. J. Nat. Cancer Inst. 39: 355-371, Aug. 1967.

Spatz, M., and Laqueur, G. L.: Evidence for transplacental passage of the natural carcinogen cycasin and its aglycone. Proc. Soc. Exp. Biol. Med. 127: 281-286, Jan. 1968.

Hirono, I., Laqueur, G. L., and Spatz, M.: Tumor induction in Fischer and Osborne-Mendel rats by a single administration of cycasin. J. Nat. Cancer Inst. 40: 1003-1010, May 1968.

Hirono, I., Laqueur, G. L., and Spatz, M.: Transplantability of cycasin-induced tumors in rats with emphasis on nephroblastomas. J. Nat. Cancer Inst. 40: 1011-1025, May 1968.

Serial No. NIAMD-LEP-2a
1. Experimental Pathology
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Inidvidual Project Report
July 1, 1967 through June 30, 1968

Project Title: Histopathologic, serum enzyme and other changes produced by X-irradiation, exercise, high altitude, cold exposures, fasting, catechol amines, dimethyl sulfoxide, various toxic compounds, and Dipetalonema witei.

Previous Serial Number: Same

Principal Investigator: Benjamin Highman

Other Investigators: Drs. P.D. Altland, H.L. Moses, B.D. Nelson and P.P. Weinstein.

Cooperating Units: LEP-NIAMD (Moses)
LPB-NIAMD (Altland, Nelson-LPB-20)
LPD-NIAID (Weinstein)

Man Years (computed for the 12 month period)
Total: ½
Professional: ½
Other: 0

Project Description:

Studies on Dimethyl Sulfoxide: Because of the unusual ability of dimethyl sulfoxide (DMSO) to act as a carrier of drugs and other agents in facilitating their passage through membranes and even intact skin, studies were initiated to determine the effect of DMSO on alterations in serum enzyme levels induced by exercise and other stresses.

In previous studies, it was shown that pretreatment of rats with DMSO, 4.5 g/kg i.p., greatly augmented a rise in certain serum enzyme values, such as glutamic oxalacetic transaminase (SGOT) and aldolase, following certain stresses such as arduous exercise or exposure to high altitude hypoxia or a cold environment. The effect of these stresses was attributed to a widespread increase in cellular permeability, and the effect of DMSO was attributed to enhancement of this increased permeability. In a current study nearing completion it was found that DMSO did not significantly augment a similar rise in serum enzyme levels following injection of epinephrine in oil. These findings suggest that the increased cellular permeability induced by epinephrine in oil is not, as was previously thought, due largely to hypoxia.

Studies on High Altitude Hypoxia: In a study with Drs. Altland

and Nelson, it was shown that ground level exercise training prior to the performance of 4 hour daily periods of exercise at high altitude significantly reduced enzyme and tissue changes and increased reproductive performance and survival. This study has been completed and is detailed elsewhere by Dr. Altland. (Drs. Altland, Highman and Nelson)

Studies on X-irradiation: In a previous study, it was found that whole body X-irradiation of rats caused a transient initial elevation in certain serum enzyme levels followed by a more prolonged fall. It was postulated that this was due to an initial increase in cellular permeability followed by a decrease. To test this hypothesis, the effect of exposure to high altitude hypoxia or a cold environment on X-irradiated rats is being investigated.

Other Studies: A study with Dr. Paul Weinstein on experimental filariasis in the Jird is nearing completion. There are no new findings to report.

Other investigations in progress include a study with Dr. Altland on the effect of DMSO on the susceptibility of rats to experimental bacterial endocarditis.

Honors and Awards:

President of the Washington Society of Pathologists.

Publications:

Garbus, Joel, Highman, B. and Altland, P. D.: Alterations in serum enzymes and isoenzymes in various species induced by epinephrine. Comp. Biochem. Physiol. 22: 507-516, 1967.

Highman, B. and Altland, P. D.: Effect of dimethyl sulfoxide, exercise, and training on rat serum enzymes and tissues. Amer. J. Physiol. 213: 779-782, Sept. 1967.

Nelson, B. D., Highman, B., and Altland, P. D.: Oxidative phosphorylation during acclimation in rats. Amer. J. Physiol. 213: 1414-1418, Dec. 1967.

Altland, P. D., Highman, B., and Nelson, B. D.: Serum enzyme and tissue changes in rats exercised repeatedly at altitude: Effects of training. Amer. J. Physiol. 214: 28-32, Jan. 1968.

Altland, P. D. and Highman, B.: Sex organ changes and breeding performance of male rats exposed to altitude: Effect of exercise and physical training. J. Reprod. Fertil. 15: 215-222, 1968.

Serial No. NIAMD-LEP-2b
1. Armed Forces Institute of Pathology
2. Radiopathology Division
3. Washington

PHS-NIH-AFIP
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:
1. Serum enzyme and pathologic changes after whole body irradiation and effect of partial shielding and protective agents.
2. Ultrastructural studies in animals given epinephrine in oil or other stresses.
3. Experimental bacterial endocarditis following X-irradiation.

Previous Serial Number: Same

Principal Investigator: Benjamin Highman

Other Investigators: Dr. D. A. Stout and Capt. A. R. Hanks (Project #1)
Dr. H. L. Moses (Project #2)
Capt. N. W. Rantanen and Capt. A. R. Hanks (Project #3)

Cooperating Units: Radiation Pathology Branch, Armed Forces Institute of Pathology (Projects #1 and #3)
Laboratory of Experimental Pathology, NIAMD, NIH (Project #2)

Man Years (computed for the 12 month period)
Total: 1½
Professional: ½
Other: 1

Project Description:

Objectives: 1. To study mechanisms contributing to changes in tissues and in serum enzyme values occurring after irradiation and other stresses.

2. To study ultrastructural changes contributing to changes in tissues and serum enzyme values occurring after various stresses, particularly after epinephrine in oil.

3. To elucidate factors causing increased susceptibility to infection following X-irradiation.

Methods Employed: 1. Various regions of the body of rats are shielded with lead during exposure to X-irradiation. Blood samples for enzyme determinations are obtained at intervals after X-irradiation.

2. Sections of heart and liver from untreated controls and rats treated with epinephrine in oil are prepared for study with the electron microscope. Some rats also receive thorotrast intravenously to

act as a tracer and aid in detecting possible alterations in vascular and cellular permeability.

3. Rats are given one or more intravenous injections of cultures of certain strains of bacteria at various intervals after whole body X-irradiation.

Major Findings: 1. Unshielded rats showed a moderate rise in plasma glutamic oxalacetic transaminase (PGOT) at 6 hours after irradiation and a marked fall in plasma alkaline phosphatase (PAkP) 4 days after irradiation. Shielding the abdomen prevented or greatly lessened these changes. Irradiating only the abdomen produced a relatively small rise in PGOT at 6 hours but caused a marked fall in PAkP at 4 days after irradiation. It is suggested that the rise in PGOT is derived from damaged lymphoid and other radiosensitive tissues and in part from a widespread initial increase in cellular permeability induced by X-irradiation. The fall in PAkP is attributed to radiation injury to the intestinal mucosa.

2. Ultrastructural changes induced by epinephrine in oil appear to vary with time and are under study.

3. Bacterial endocarditis has been induced in X-irradiated rats by intravenous injection of cultures of Streptococcus mitis. Factors influencing the incidence of endocarditis in X-irradiated rats are under study.

Research Significance: 1. Study of plasma enzymes changes may aid in elucidating the mechanism of action of X-irradiation and other stresses and the origin of the altered serum enzyme levels. Our findings indicate that X-irradiation may cause an increase in cellular permeability followed by a decrease. X-irradiation, therefore, may serve as a model, utilizing drugs and other compounds, in studying factors that affect cellular permeability and the maintenance of normal blood values.

2. It has been postulated that the serum enzyme changes following administration of epinephrine in oil are due to an altered cellular permeability. Ultrastructural studies may aid in substantiating this hypothesis and in determining the nature of such altered permeability.

3. The resistence to certain infections is lowered by X-irradiation, but the mechanisms involved are not fully understood. The induction of bacterial endocarditis in X-irradiated animals may serve as a model in clarifying some of the factors causing the reduced resistence to infection.

Proposed Course of Project: 1. This project is nearing completion and suggests that the abdominal organs, particularly the intestine, play a major role in accounting for the plasma enzyme changes following X-irradiation.

2. Some rats have been given thorotrast intravenously. This substance is readily detected with the electron microscope and may aid in determining possible alterations in vascular and cellular permeability induced by epinephrine in oil and other stresses.

3. The effect of protective agents and partial shielding on the susceptibility of X-irradiated rats to bacterial endocarditis is under study.

Serial No. NIAMD-LEP-3
1. Experimental Pathology
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1. Electron microscope autoradiographic localization of H^3-cholesterol in rat adrenal cortex with and without ACTH stimulation.
2. Ultrastructural studies of continuous cell lines derived from peripheral blood of patients with infectious mononucleosis.
3. Significance of cellular alterations in rat liver with allylisopropyl acetamide induced hepatic porphyria.

Previous Serial Number: Same

Principal Investigator: Harold L. Moses

Other Investigators: Drs. W. W. Davis, A. S. Rosenthal, L. Garren, L. N. Chessin, P. R. Glade, R. G. Suskind, D. P. Tschudy, J. A. Kasel.

Cooperating Units: LEP-NIAMD (Suskind)
LKEM-NHI (Rosenthal)
LCI-NIAID (Chessin, Glade & Kasel)
MET-NCI (Tschudy)

Dr. W. W. Davis (Department of Physiology, Hershey Medical Center, Hershey, Pa. - formerly CE-NHI)
Dr. L. Garren (Department of Medicine, Yale University, New Haven, Conn. - formerly LCB-NIAMD)

Man Years (computed for the 12 month period)
Total: 2
Professional: 1
Other: 1

Project Description:

1. Studies on the mechanism of ACTH stimulation of adrenal steroidogenesis have been continued during the past year. Previous studies had shown that routine procedures for processing tissue for electron microscopy resulted in extraction of over two-thirds of the tritium-labeled cholesterol from rat adrenal cortex. A method of processing was developed which allowed retention of up to 90 per cent of H^3- cholesterol and permitted autoradiographic studies at the electron microscope level of corticosteroid-precursor cholesterol pools in rat adrenal cortical cells. Approximately 70 per cent of developed grain was found to be over the lipid droplets in

the hypophysectomized control animals. The relative cross-sectional area of lipid droplets was 8 per cent, and the specific activity of lipid droplets was 2.25 grains/μ^2, whereas the cytoplasm had 0.05 grains/μ^2 with the grains evenly distributed between the mitochondria and other cytoplasmic areas. With ACTH stimulation, there was depletion of counts from all cell fractions, but the bulk of the depleted grain was from the lipid droplet fraction. This depletion was blocked by cycloheximide which caused a slight increase in specific activity in the non-lipid droplet, non-mitochondrial cytoplasm.

Biochemical studies were also carried out for correlation with the electron microscope autoradiographic data. A method of obtaining purified fractions of adrenal cortical cell lipid droplets was devised. Determinations of the distribution of relative counts in free and esterified cholesterol in the purified lipid droplet fraction and in cell fractions obtained by routine procedures of differential centrifugation were in general agreement with the relative distribution of counts as determined by electron microscope autoradiography. Corticosterone production and specific activity of tritium label was determined on the various experimental groups _in vivo_ and in pre-labeled adrenal slices incubated _in vitro_ in the presence and absence of ACTH and/or cycloheximide.

The data obtained were consistent with the following interpretations: (1) Exogenously administered cholestrol was incorporated into the adrenal cortical cell lipid droplet in esterified form. (2) The lipid droplet cholesterol probably represented a major pool of steroid precursor cholesterol. (3) ACTH stimulation caused conversion of esterified to free cholesterol which was transported into mitochondria and converted to pregnenolone and through subsequent steps to corticosterone. (5) Cycloheximide blocked the cholesterol-to-pregnenolone step and resulted in accumulation of the metabolic intermediate (free cholesterol) in the soluble cell fraction as determined by ultracentrifugation or in the non-lipid droplet, non-mitochondrial cytoplasm as determined by autoradiography. It could not be determined, however, whether cycloheximide blocked transport of free cholesterol into the mitochondria or inhibited a metabolic step within mitochondria with rapid back-diffusion of the unmetabolized cholesterol. (Drs. Davis, Rosenthal, and Garren).

2. Continuous suspension cultures derived from peripheral blood leukocytes of patients with infectious mononucleosis have been established (Drs. Glade and Chessin). Under identical conditions, peripheral blood leukocytes from control individuals did not proliferate in long-term culture. Electron microscopic studies of fourteen cell lines derived from infectious mononucleosis patients has been carried out. These cells in culture had a lymphoblastoid appearance. The herpes-like virus (HLV) was identified in four of the fourteen cell lines examined. The HLV has been identified by other investigators in lymphoid and myeloid cell lines derived from patients with a variety of disorders, including Burkitt's lymphoma, leukemia, a variety of malignancies, and apparently healthy

individuals. A far more consistent structural finding in the infectious mononucleosis cell lines was the presence of reticular aggregates of 22 mμ particles present in association with the rough endoplasmic reticulum. These dense aggregates were found in a relatively high percentage of cells in all of the fourteen cell lines examined. Similar structures have been described previously in meningioma and meningeal sarcoma cells induced in dogs by Rous sarcoma virus, in sarcoma cells induced in monkeys by the Rous virus, in cell lines derived from Burkitt's lymphoma and in cultured monkey kidney cells infected with rubella virus. Also present in the infectious mononucleosis cell lines were small (25-50mμ) extracellular virus-like particles which were morphologically distinct from the HLV. These small extracellular particles were more numerous in those four cell lines which contained both the HLV particles and the cytoplasmic reticulum of 22 mμ particles. Attempts to transmit infective particles from these cell lines, however, have so far been unsuccessful. Studies are currently under way to determine if the detected virus-associated or virus-like particles are indeed related to a transmissible agent which may be involved in the pathogenesis of infectious mononucleosis and the ability of peripheral blood leukocytes to survive in long-term culture. (Drs. Chessin, Glade and Kasel)

3. Previous studies in this laboratory have shown that administration of allylisopropyl acetamide (AIA) to rats causes hepatocellular hypertrophy accompanied by a marked increase in the amount of smooth endoplasmic reticulum and by prominent nucleolar enlargement. AIA, which is chemically related to the barbituates, is a porphyrinogenic compound and produces an experimental disease similar to human acute intermittent porphyria. The mechanism of increased porphyrin production in both the human and experimental porphyria is thought to be increased activity of the rate-limiting enzyme in heme biosynthesis, delta-aminolevulinic acid synthetase. That the increased enzyme activity with AIA administration is due to enzyme induction rather than simple enzyme activation is indicated by the observation that inhibitors of protein synthesis block the increase in enzyme activity. Phenobarbital causes the induction in rat liver of a number of enzymes accompanied by hypertrophy of the smooth endoplasmic reticulum, but does not cause increased porphyrin production. A comparative study of the ultrastructural changes accompanying the states of enzyme induction with AIA or phenobarbital administration is in progress. It is hoped that this comparative study in conjunction with studies utilizing adrenalectomy or inhibitors of protein syntheses (Actinomycin D or puromycin) will allow differentiation of changes associated with drug detoxification from those accompanying enzyme induction and increased porphyrin production. (Drs. Suskind and Tschudy)

Publications:

Epstein, C. J., Moses, H. L., Epstein, L. B., and Garrison, M. M. A structural analysis of hepatomegaly induced by a hormone-secreting tumor. Exp. Molec. Path. 7: 304-326, Dec. 1967.

Moses, H. L., and Rosenthal, A. S. On the significance of lead-catalyzed hydrolysis of nucleoside phosphates in histochemical system (Letter to the Editor). J. Histochem. Cytochem. 15: 354-355, June, 1967.

Ganote, C. E., Grantham, J. J., Moses, H. L., Burg, M. B. and Orloff, J. Ultrastructural studies of vasopressin effect on isolated perfused renal collecting tubules of the rabbit. J. Cell Biol. 36: 355-367, Feb. 1968.

Glade, P. R., Kasel, J. A., Moses, H. L., Whang-Peng, J., Hoffman, P. F., Kammermeyer, J. K., and Chessin, L. N. Infectious mononucleosis: Continuous suspension culture of peripheral blood leukocytes. Nature 217: 564, Jan. 1968.

Ganote, C. E. and Moses, H. L. Light and dark cells or artifacts of liver fixation. Lab. Invest. (In press).

Moses, H. L., Glade, P. R., Kasel, J. A., Rosenthal, A. S., Hirshaut, Y., and Chessin, L. N. Infectious mononucleosis: Detection of herpes-like virus (HLV) and reticular aggregates of small cytoplasmic particles in continuous lymphoid cell lines derived from peripheral blood. Proc. Nat. Acad. Sci. (In press).

Chessin, L. N., Glade, P. R., Kasel, J. A., Moses, H. L., Hirshaut, Y., and Heberman, R. Combined Clinical Staff Conference of the National Institutes of Health. The circulating lymphocyte - its role in infectious mononucleosis. Ann. Intern. Med. (In press).

Moses, H. L. and Rosenthal, A. S. Pitfalls in the use of lead ion for histochemical localization of nucleoside phosphatosis. J. Histochem. Cytochem. (In press).

Serial No. NIAMD-LEP-4
1. Experimental Pathology
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on transfer ribonucleic acid synthesis.

Previous Serial Number: Same

Principal Investigator: David W. E. Smith

Other Investigators: Dr. Thomas W. Tillack

Cooperating Units: LEP-NIAMD

Man Years (computed for the 12 month period)
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

There has recently been considerable interest in the possibility that changes in the relative amounts of different transfer RNA (tRNA) species, formation of special tRNA molecules, and modifications in the structure of certain tRNA molecules may have some role in the genetic control of protein synthesis. It is also of interest whether the same pool of tRNA molecules is used for the synthesis of all proteins made by a cell, or whether alterations in this pool occur depending on which proteins are being made.

We have completed development of a method for the study of tRNA synthesis by pulse labeling tRNA and fractionating it on a column. Radioactive guanine is used, and there is evidence that guanine incorporation reflects the DNA dependent portion of tRNA synthesis. In control experiments in which doubly labeled samples of tRNA from similarly treated cultures of E. coli K12 were fractionated, the ratio of the isotopes throughout elution from the column remained constant.

tRNA synthesis was studied during the first five minutes following induction of the lactose operon in these E. coli. Induction of the enzymes of this operon offers an especially uncomplicated system for investigation of tRNA synthesis because the induction is not accompanied by changes in the doubling time or substrate utilization of the bacteria which may cause changes in tRNA synthesis which are independent of the synthesis of the specific enzymes induced. No difference was found between induced and control cells in our experiments. It is estimated that the synthesis of a new tRNA species accounting for 2 percent or more of the tRNA synthesized during the period of labeling could have been detected. Our results suggest that the composition of the pool of tRNA species remains unchanged following induction despite the significant alteration in protein synthesis that occurs.

tRNA synthesis has also been investigated during the initial five minutes of T2 bacteriophage infection of *E. coli* in collaboration with Dr. Thomas W. Tillack (see NIAMD-LEP-11). The pattern of tRNA synthesis in infected cells is markedly different from that in control cells.

Publications:

Smith, D.W.E.: The measurement of transfer ribonucleic acid synthesis during induction of the lactose operon. *J. Biol. Chem.* 243: 1968.

Serial No. NIAMD-LEP-5
1. Experimental Pathology
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Autoradiographic and cytochemical studies regarding structure and function of the nucleolus.

Previous Serial Number: Same

Principal Investigator: R. Gerald Suskind.

Other Investigators: Drs. H. L. Moses, G. Rabotti and Mr. T. Pry.

Cooperating Units: LEP-NIAMD (Moses)
LVO-NCI (Pry)
Laboratoire de Medicine Experimentale, College de France, Paris (Rabotti)

Man Years (computed for the 12 month period)
Total: 1
Professional: 1

Project Description:

The synthetic functions of the nucleolus in the early stages of Rous Sarcoma virus infection have been investigated. The cellular pathology underlying the "neoplastic" transformation of Rous Sarcoma virus infected cells remains poorly understood and compared with that of the small RNA viruses little is known about the site and manner of intracellular viral RNA synthesis. Studies with metabolic inhibitors of RNA synthesis, such as Actinomycin, and inhibitors of DNA and protein synthesis, have shown a complex interdependence of infectivity and transformation with cellular DNA and protein synthesis varying with the time of administration of the inhibitors; but have failed to clarify convincingly the mechanism or the site of viral RNA synthesis. Conflicting reports concerning inhibition of intracellular viral RNA by Actinomycin may be due in part to the complexity of physical separation of a small amount of rapidly degraded viral RNA in a large excess of host RNA. Using quantitative autoradiographic techniques experimental data has been obtained which support the interpretation that the nucleolus is a site of synthesis or assembly of viral RNA.

Low doses of Actinomycin selectively inhibit nucleolar RNA synthesis probably as a result of a greater affinity for the G-C rich template sites of ribosomal precursor RNA. Previous investigations have shown that a concomitantly labeled protein assembled or synthesized in the nucleolus is also selectively inhibited by Actinomycin. It was found, however, that these effects, as well as the delayed inhibition of DNA synthesis are reversible by removal of the inhibitor. By studying the rate of this reversal significant and consistent differences in nucleolar and nuclear labeling per unit area were found between infected and uninfected cells and their respective controls.

Secondary cultures of RIF free chicken fibroblasts were treated for 3 hours with 0.1 or 0.2 μgm/ml of Actinomycin, and were washed and infected with Bryan strain RSV in a multiplicity of about 100. Control cells were either mock-infected in the suspending medium or exposed to a heat-inactivated virus suspension. At varying times following infection the cells were then pulse labeled with tritiated uridine for 10 min. so that the label appeared only in the nucleus. Differential grain counts of the nucleolus and nucleus were made in radioautographs prepared as previously reported, using groups of 25 randomly selected cells. Statistical comparisons by standard t-test were made of the separate mean grain counts, grain counts per unit area, and of the means of the ratios in the nucleolus and nucleus using computer analysis of the raw data.

It was found that nucleolar RNA synthesis is transiently increased after Actinomycin inhibition in cells subsequently infected when compared with infected cells not previously inhibited and with respect to uninfected control cells. This relative increase of nucleolar RNA synthesis occurs around 10 hours post infection, disappearing by 24 hours and precedes an equivalent rise in infected cells not previously inhibited. These effects are not observed with the heat-inactivated virus inoculum. The labeling of ribosomal protein in the nucleolus, using a short pulse of tritiated lysine, shows no such increase after virus infection and is identically inhibited by Actinomycin in infected and uninfected cells. During the time that nucleolar RNA synthesis is increased in infected cells DNA synthesis is identically inhibited in infected and uninfected cells. However, shortly before the appearance of morphologic transformation the rate of DNA synthesis is faster in infected control cells than in Actinomycin-treated infected or uninfected cells.

The findings that increased synthesis of nucleolar RNA in RSV-infected cells occur under conditions in which nucleolar ribosome synthesis is selectively inhibited support the interpretation that the nucleolus is a site of synthesis or assembly of a viral RNA not inhibited by Actionmycin. The dissociation of this nucleolar RNA label from nucleolar ribosomal protein also strongly suggests that this label is a viral RNA separate from host nucleolar ribosomes. It is proposed that under these experimental conditions a viral RNA may compete with Actinomycin for some G-C rich template sites of ribosomal precursor RNA in the nucleolus. However the possibility that the nucleolar protein inhibited by Actinomycin may act as an interferon-like agent, inhibition of which could accelerate nucleolar RNA synthesis in infected cells is under experimental consideration. Inhibition of cellular DNA synthesis by Actinomycin and resultant differences in the rate of increase prior to transformation probably account for the partial depression by low doses of Actinomycin of the incidence of transformation which appears to be unrelated to inhibitions of viral RNA.

In collaboration with Dr. Harold Moses electron microscopic and radiochemical studies on the ultrastructural sites of synthesis of ribosome associated protein in the nucleolus are in progress. Previous investigations on the effect of temperature of aldehyde fixation on nucleolar RNA and protein labeling have shown that fixation at 37° C results in a dissociation of nucleolar protein and RNA label and in a reduction of protein label in the nucleolus identical to that incurred by Puromycin or Actinomycin inhibition. These findings suggested the interpretation that the proportion of nucleolar protein not fixed at 37° C is associated with ribosomal RNA. Preliminary results of radiochemical studies in parallel with electron microscopic radioautography confirm that significant elution of amino acid label occurs in glutaraldehyde fixation at 37° C and that the fibrillar elements of the nucleolus appear to be the major source of this protein.

Publications:

Suskind, R. G.: Effect of temperature of aldehyde fixation on the radioautographic localization of ribonucleoprotein in nucleoli of hela cells. J. Cell Biol. 34: 721-734, September 1967.

Serial No. NIAMD-LEP-6
1. Experimental Pathology
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1. Pathways of water transport across the renal collecting tubule.
2. Comparative pathology of hepatotoxic agents.
3. Effects of fixation on hepatic ultrastructure.

Previous Serial Number: Same

Principal Investigator: Charles E. Ganote, M.D.

Other Investigators: Dr. J. Grantham

Cooperating Units: LKEM-NHI

Man Years (computed for the 12 month period)
Total: 2
Professional: 1
Other: 1

Project Description:

1. In a previous study it was shown that interspaces between cells, in the isolated perfused renal collecting tubule, dilate during vasopressin-induced osmotic water flow. This suggested that the interspaces may have a role as part or all of the pathway for water flow across the tubule. The present study has further elucidated the role of the lateral interspaces in bulk water flow and has established beyond reasonable doubt that water does enter the space across the lateral cell membranes and exits via the basal slit or pore between the cells. Oubaine added to transporting tubules stops the short circuit current but does not affect the dilatation of the space indicating that salt is not transported into the space. Swelling is also not due to trapped solute because the spaces do not dilate when tubules are swollen in a hypotonic medium. The placing of control or swollen tubules in a hypertonic or isotonic medium produces large intercellular spaces. This could occur only if the basal slit were patent to water and solute and water entered into the space across the lateral cell membranes, since the basement membrane is not a rigid structure. Further evidence of the patency of the pore is provided by separation of the cell membranes and the entrance of electron microscopic tracers into the intercellular space even though the tracers do not penetrate into the region of the tight apical junction. The rate of emptying of the intercellular spaces was shown to be directly dependent on the intraluminal hydrostatic pressure which in turn depended on the elastic properties of the basement

membrane. Alternatively with the shrinkage of cells to a point where the basement membrane offered no positive pressure the dilated spaces did not collapse with time. Evidence that water flows across the basal cell membrane as well as across the lateral cell membranes was obtained by observations that the shrinkage of tubules swollen to different degrees was always biphasic with an initial rapid rate due to movement of water across the basal cell membrane and a long slower expodential rate due to emptying of the intercellular spaces. Preliminary calculations suggest that the majority of water flows across the basal cell membrane but that a significant portion also flows through the intercellular space.

2. There has been general agreement among pathologists that hepatic cells respond in an undifferentiated and nonspecific manner to toxic injury by a wide variety of hepatotoxins. This thesis was tested and found to be incorrect by a direct comparative study of the ultrastructural pathology induced by three biochemically distinct classes of hepatotoxins. Two alkylating agents (MAM and DMNA), CCl_4 and hydrazine sulfate were used. It was found that the two alkylating agents produced similar lesions consisting of nucleolar microsegregation, hypertrophy of the SER, formation of membrane whorls, decreases in the number of ribosomes and endothelial damage. The CCl_4-induced changes were quite distinct and consisted of formation of disorganized membrane clumps, increased mitochondrial density, depletion of membranes and vacuolization of the Golgi apparatus. Hydrazine caused changes entirely distinct from the other agents. These changes were nuclear and nucleolar enlargement, prominent mitochondrial swelling and increased lipid in the Golgi membranes. It was concluded that ultrastructural liver pathology is relatively specific for different biochemical classes of hepatotoxic agents and that the ultrastructural changes are a direct reflection of the biochemical changes in the cells.

3. A study was conducted of the effects of different fixation techniques on liver ultrastructure. It was found that a wide variety of hepatic changes could be induced by the fixation techniques now in general use. These artifacts of liver fixation were often similar to those changes reported in the literature as nonspecific normal or pathological lesions. They included a variety of mitochondrial changes, dilatation and vesiculation of the endoplasmic reticulum, clumping of nuclear chromatin and variations of cellular density with formation of light and dark cells. This study stressed the need for rapid and complete fixation of liver cells in order to preserve hepatic ultrastructural details. An improved method of liver fixation was developed using portal vein perfusion at high flow rates (~70 ml/min/100 gm body weight) with a nearly isotonic glutaraldehyde solution. This technique has been used with consistently good results in several ultrastructural studies.

Publications:

Ganote, C. E., Grantham, J. J., Moses, H. L., Burg, M. B. and Orloff, J.: Ultrastructural studies of vasopressin effect on isolated perfused renal collecting tubules of the rabbit. J. Cell Biol. 36: 355-367, Feb. 1968.

Ganote, C. E. and Moses, H. L.: Light and dark cells as artifacts of liver fixation. (Accepted for publication in Lab. Invest.)

Serial No. NIAMD-LEP-7
1. Experimental Pathology
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Preparation of stained tissue sections for investigation and diagnostic purposes.

Previous Serial Number: Same.

Principal Investigator: Mr. Walter Rawlings - Head, Tissue Preparation Laboratory.

Other Investigators: None.

Cooperating Units: None.

Man Years (computed for the 12 month period)
Total: 5
Professional: 0
Other: 5

Project Description:

The statistical report of this unit is shown below. In addition to preparing material for projects conducted in the Laboratory of Experimental Pathology, many other investigators received advice and service. These include: Drs. Black and Rowe - Laboratory of Infectious Diseases; Drs. Weinstein, Richards and Cheever - Laboratory of Parasitic Diseases; Dr. Barth, Arthritis and Rheumatism Branch. Tissue from a small number of animals were prepared for 12 other investigators.

	Specimens Accessioned	Stained Slides		Spare Slides	Total
		Routine	Special		
Animals*	4,265	5,833			
Surgicals	2,359	3,624			
Autopsies	11	77			
		9,534	8,147	19,109	36,790

*Source of animal material:

NIAMD	2185
Other Institutes	2080

Serial No. NIAMD-LEP-8
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Histochemistry: principles, methods and applications.

Previous Serial Number: None.

Principal Investigator: Ned Feder

Other Investigators: Dr. Lois Tice, Dr. Bruce K. Wetzel and Dr. Fotis C. Kafatos.

Cooperating Units: NIAMD-LEP (Tice and Wetzel)
Dept. of Biology, Harvard University (Kafatos)

Man Years (computed for the 12 month period)
Total: 3
Professional: 1
Other: 2

Project Description:

Cytodifferentiation in the Maxillary Galea. The maxillary galea of certain saturniid moths is a particularly favorable organ for the study of development, because of its highly specialized synthetic activity, abrupt and predictable start of specialized synthesis, and ease of study in all developmental stages. A report on the development of this organ has been submitted for publication. The work was carried out with Dr. F. C. Kafatos.

Hydrophilic Embedding Media for Lipid and Enzyme Histochemistry. Work on the development and application of new histochemical methods is in progress, this work is a continuation of that carried out at Harvard University over the past 6 years. With hydrophilic embedding media, particularly glycol methacrylate, one can under suitable conditions preserve and demonstrate lipids and enzymes that ordinarily are lost or destroyed (Feder, J. Cell Biol. 19: 23A, 1963); at the same time the preservation of tissue and cell structure is excellent as revealed by light and electron microscopy. A report on the use of glycol methacrylate has appeared recently (Feder and O'Brien, Amer. J. Botany 55: 123-142, 1968); studies on the tissues of humans and laboratory animals are under way.

"Microperoxidases" as Histochemical Markers. I have found that certain heme-peptides with molecular weights in the range 900 to 2000 have sufficiently high peroxidase activity so that they can be demonstrated histochemically at the level of the light and electron microscope. These "microperoxidases", because of their small size, have advantages over horseradish peroxidase, molecular weight about 40,000, which has been used as a marker for antibodies (Nakane and Pierce, J. Cell Biol. 33: 307-318, 1967) and as a means of delineating channels of tissue fluid movement (Karnovsky, J. Cell Biol. 35: 213-236, 1967).

Cesium Polycarboxylates for Density Gradient Ultracentrifugation. Some studies, in collaboration with F. C. Kafatos, on buoyant media for the ultracentrifugal separation and analysis of nucleoproteins are nearing completion. We have demonstrated the value of certain cesium polycarboxylates for ultracentrifugal analysis. We have suggested a mechanism explaining (a) the low buoyant density of nucleic acids and nucleoproteins in concentrated solutions of these compounds, and (b) the superiority of these compounds to cesium chloride in their denaturant effects on the ribosomes of higher animals.

Serial No. NIAMD-LEP-9
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunochemical studies with antigenic substances and their localization in tissues with special reference to prolactin.

Previous Serial Number: NIAMD-LEP-8

Principal Investigator: Dr. E. W. Emmart

Other Investigators: Drs. R. W. Bates, A. E. Wilhelmi, J. M. Mossakowski.

Cooperating Units: LNE-NIAMD (Bates)
Emory University, Dept. of Biochemistry (Wilhelmi)
Polish Academy of Sciences, Warsaw (Mossakowski)

Man Years (computed for the 12 month period)
Total: 2
Professional: 1
Other: 1

Project Description:

Studies with prolactin. A study dealing with the localization of prolactin in cultured cells of the rostral pars distalis of the pituitary of Fundulus heteroclitus (Linnaeus) was completed and published showing localization of prolactin by means of fluorescent antibody to ovine prolactin.

A second study is in progress which describes the behavior and morphology of cultured cells of the rostral pars distalis under high magnification and at successive intervals.

An immunochemical study with prolactin-like fractions of fish pituitaries in collaboration with A. E. Wilhelmi was completed and the paper was accepted for publication in General and Comparative Endocrinology. An additional study describing the use of immunochemical binding and sephadex filtration for purification of ovine and piscine prolactin in collaboration with R. W. Bates has been completed and has been submitted for publication.

Work on the localization of prolactin in the pituitary of Carassius has begun. The morphology of this fish is similar to that of the Pacific Salmon and therefore of particular interest. It is also the only fish beside the African lung fish, in which the fish-prolactin is reported to cause typical mammotrophic reaction in the pigeons.

Publications:

Emmart, E. W. and Mossakowski, M. J.: The localization of prolactin in cultured cells of the rostral pars distalis os the pituitary of *Fundulus heteroclitus*, Linnaeus. *Gen. Comp. Endocr*. 9: 391-400, Dec. 1967.

Emmart, E. W. and Wilhelmi, A. E. Immunochemical studies with prolactin-like fractions of fish pituitaries. *Gen. Comp. Endocr*. (accepted for publication).

Serial No. NIAMD-LEP-10
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Ultrastructural and cytochemical studies including: the development of rabbit myelocytes and their cytoplasmic granules; the localization of various phosphatases in bacteria; the effect of parathyroidectomy on parafollicular cells in rat thyroid glands; a descriptive survey of "foamy follicles" in mouse thyroids; and studies of normal and regenerating planaria.

Previous Serial Number: NIAMD-LEP-9

Principal Investigator: Bruce K. Wetzel

Other Investigators: H. F. Dvorak, R. F. Gittes, L. A. Heppel, S. S. Spicer, S. Wells and S. H. Wollman.

Cooperating Units: Massachusetts General Hospital, Boston, Mass. (Dvorak and Gittes)
Cornell University, Ithaca, N. Y. (Heppel)
Duke University, Chapel Hill, N. C. (Wells)
LPHY-NCI (Wollman)
University of S. C., Charleston, S. C. (Spicer)

Man Years (computed for the 12 month period)
Total: 2
Professional: 1
Other: 1

Project Description:

Cytochemical studies of rabbit granulocytes have been completed. These studies will now be extended to include other laboratory animals and humans for a general review of granulocyte development. Autoradiographic studies of eosinophil granule maturation are contemplated.

Studies on the localization of phosphatases in E. coli have been concluded, providing evidence for the presence of cyclic phosphodiesterase glucose-6-phosphatase, and alkaline phosphatase in the periplasmic space of these bacteria. The presence of these enzymes in the periplasmic compartment is consistent with biochemical data, and focuses attention on a space hitherto regarded by morphologists as an artifactual consequence of protoplast shrinkage (Drs. Spicer, Heppel and Dvorak). We have also demonstrated these enzymes in the periplasm of a "minicell" strain of E. coli, which buds off tiny, terminal anucleate fragments. Drs. Heppel and Dvorak have shown biochemically that these fragments contain very high concentrations of the periplasmic enzymes. Together, these findings verify the existence of the periplasmic space and support the cytochemical localization of phosphatases in it.

In thyroid glands of normal C3H mice we have characterized by electron microscopy a second system of follicles in addition to the usual functional follicles. Follicles of this second system contain typical epithelial cells and parafollicular cells which are similar to those of usual follicles, but are distinguished by 1) a heterogeneous, "foamy" colloid, 2) occasional ciliated epithelial cells with long microvilli and sparsely populated cytoplasm, and 3) a newly described atypical epithelial cell characterized by abundant agranular reticulum and occasionally by glycogen and secretory globules of unknown nature. This second type of follicle is of interest as a possible derivative of the lateral or ultimobranchial contribution to the developing gland. Developmental studies of embryonic mouse thyroid have been initiated to explore this possibility (Dr. Wollman).

An extensive histological study of thyroid glands from long-term parathyroidectomized rats has been terminated. A marked (>3 fold) hyperplasia of parafollicular cells is evident in animals with chronic hypercalcemia. This effect is not observed in animals receiving protective parathyroid implants. Thyrocalcitonin is known to accumulate in parathyroidectomized rats, thus implicating the parafollicular cell in thyrocalcitonin synthesis and storage. This work, including complementary ultrastructural studies, comparison of contralateral lobes, and the evaluation of several thyroids from hypophysectomized-parathyroidectomized rats, is being readied for publication (Drs. Gittes and Wells).

Descriptive fine structural studies of normal planarian tissues have been resumed. The protonephridial system has been found to comprise four distinct segments: 1) terminal flame cells with pores communicating with the intercellular space, 2) subsequent tubules with cilia arising sporadically from their inner surfaces, leading to 3) non-ciliated segments with elaborately infolded basal surfaces, and 4) simple ducts which presumably lead to the exterior. Also, the complex process of rhabdite formation has been reconstructed morphologically. These findings will serve for comparison with regenerating tissues.

Publications:

Spicer, S. S., Staley, M. W., Wetzel, M. G., and Wetzel, B. K: Acid mucosubstances and basic protein in mouse paneth cells. J. Histochem. Cytochem. 15: 225-242, Apr. 1967.

Wetzel, B. K., Spicer, S. S. and Horn, R. G.: Fine structural localization of acid and alkaline phosphatases in cells of rabbit blood and bone marrow. J. Histochem. Cytochem. 15: 311-334, June 1967.

Serial No. NIAMD-LEP-11
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1) Isolation and characterization of protein components of animal cell membranes.
2) The effect of bacteriophage T2 infection of *Escherichia coli* on the synthesis of transfer RNA.

Previous Serial Number: NIAMD-LEP-10

Principal Investigator: Thomas W. Tillack

Other Investigators: Drs. V. T. Marchesi, D.W.E. Smith, and E. Steers, Jr.

Cooperating Units: NCI-GL&C (Marchesi)
NIAMD-LCB (Steers)
NIAMD-LEP (Smith)

Man Years (computed for the 12 month period)
Total: 1
Professional: 1
Other: 0

Project Description:

1) Approximately one-half of the dry mass of most animal cell membranes consists of protein, and little is known about its chemical composition and physical properties. Marchesi and Steers (*Science* 159: 203, 1968) have found that approximately 20 percent of the membrane-bound protein of guinea pig erythrocyte ghosts can be solubilized and obtained free of bound lipid, carbohydrate and nucleic acid by dialysis against adenosine triphosphate and 2-mercaptoethanol. The solubilized protein fraction appears to consist principally of one protein, which may have a structural role in the intact red blood cell membrane. It has been characterized by polyacrylamide gel electrophoresis, free-boundary electrophoresis, analytical ultracentrifugation, immunoprecipitin reactions, amino acid analysis, and by electron microscopy using negative staining techniques and metal shadowing.

We have found that this protein is present in all species of red blood cells so far examined--human, horse, rabbit, sheep, rat, and guinea pig. This protein can be extracted from red cell membranes of all these species by treatment of the membranes with low ionic strength media containing chelating agents and sulfhydryl compounds and purified by salt fractionation and gel filtration with Sephadex and Sepharose column chromatography. The soluble proteins of all species of red cells appear to be identical by several criteria including gel electrophoresis and immunoprecipitin reactions. Analytical ultracentrifugation studies indicate that the proteins are homogeneous and have a fibrous structure. Amino acid analyses of this protein from several species show striking similarities.

We plan to apply similar methods to studies of the protein components of membranes of nucleated cells and to start isolating and extracting cell membrane preparations of tissue culture cells. These studies of erythrocyte membranes will also provide ways to study the chemical basis for certain diseases of red blood cells, such as hereditary spherocytosis. Recently we have begun attempts to extract and study the remaining proteins of the erythrocyte membrane, many of which are complexed to carbohydrates and lipids (Tillack, Marchesi and Steers).

2) The metabolic alterations which result from T-even bacteriophage infection of E. coli include cessation of host DNA, RNA, and protein synthesis and the synthesis of DNA, messenger RNA, and new proteins specific to the phage. There is also evidence that at least one species of transfer RNA, leucyl-tRNA, is altered after bacteriophage infection of E. coli (J. Molec. Biol. 20: 183, 1966). The purpose of our investigation was to determine whether new synthesis of tRNA occurs after T2 infection of E. coli B, and if so, whether the pattern of synthesis is different from that in uninfected cells. Any modification in the amount or structure of the species of tRNA synthesized after phage infection might be important in controlling the switch-over from bacterial to phage protein synthesis, since the translation of phage messenger RNA might be expected to require or to utilize preferentially species of tRNA synthesized in the presence of the phage.

We found that during the initial five minutes of T2 bacteriophage infection of E. coli, there is substantial incorporation of labeled guanine into the transfer RNA fraction, strongly suggesting that new tRNA is synthesized during this early stage of phage infection. In this five-minute period the amount of labeled guanine incorporated into the tRNA fraction of T2-infected bacteria is 23 percent of that in uninfected bacteria during an equivalent period.

The tRNA fractions from T2-infected bacteria and uninfected bacteria were compared by pulse-labeling each of the two cultures for five minutes with ^3H and ^{14}C isotopes of guanine and partially resolving a mixture of the doubly-labeled tRNA species by reversed phase chromatography. The chromatographic pattern of the labeled tRNA from uninfected cultures of E. coli had a chromatographic pattern that was radically different from that of tRNA prepared from uninfected cultures. These results indicate either that the tRNA synthesized after T2 infection of E. coli represents species of tRNA entirely different from those made in uninfected bacteria, or that relative proportions of the different species made are greatly altered after phage infection, with some being made preferentially and others in reduced amounts (Tillack and Smith).

Publications:

Tillack, T. W. and Smith, D.W.E.: The effect of bacteriophage T-2 infection of *Escherichia coli* on the synthesis of transfer ribonucleic acid. (Accepted for publication in Virology.)

Couse, N. L., Tillack, T. W. and Korn, D.: Inhibition of bacteriophage λvir maturation by actinomycin D. (Accepted for publication in Journal of Molecular Biology.)

Serial No. NIAMD-LEP-12
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on adenosinetriphosphatase.

Previous Serial Number: None.

Principal Investigator: Lois W. Tice

Other Investigators: Drs. Ned Feder and Alan Rosenthal

Cooperating Units: LEP-NIAMD (Feder)
LKEM-NHI (Rosenthal)

Man Years (computed for the 12 month period)
Total: 1
Professional: 1
Other: 0

Project Description:

The effects of lead on histochemical ATPase systems were studied. It was found that:

1) The stability constant of lead-ATP complexes is of the same order of magnitude as ATP complexes with calcium and magnesium. Pb competes with Mg for ATP when both cations are present. When ATP is present in excess, free Pb is significantly reduced.

2) With two microsomal ATPases (liver and kidney) enzyme inhibition by lead can be largely overcome by increasing ATP concentrations--whether by reversing the effects of competitive inhibition or by chelation effects, or both, is not clear.

3) Precipitation of lead phosphate is affected by the quantity of ATP present in the medium as well as by Ca or Mg added as activating cations. At the same time, significant quantities of nucleotide substrate may be lost to precipitate.

The results of these biochemical studies of ATPase have a direct bearing on interpreting histochemically demonstrated ATPase. The results can also be applied to the development of improved methods for the histochemical demonstration of this enzyme.

Honors:

Secretary-treasurer, Washington Society for Electron Microscopy, 1967-
Associate Editor of new Journal, Tissue and Cell.

Publications:

Tice, Lois W.: *In situ* studies of the sarcotubular system. In Tanz, Ralph G. (Ed.): *Factors Influencing Myocardial Contractility*. New York, N.Y., Academic Press, 1967, pp. 339-350.

Tice, Lois W.: A comparison of the distribution of enzymatically and nonenzymatically produced lead phosphate in insect flight muscle. (Accepted for publication in Tissue and Cell.)

Serial No. NIAMD-LEP-13
1. Experimental Pathology
2. Rheumatic Diseases
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title : Pathogenesis of experimental arthritis and pathology of rheumatism.

Previous Serial Number: NIAMD-LEP-11

Principal Investigator: Leon Sokoloff, M.D.

Other Investigator : William T. Green, Jr., M.D.

Cooperating Units : Edward D. Eanes, Ph.D. (LHP-NIDR)
Garland N. Martin, B.A. (LHP-NIDR)
Katharine C. Snell, M.D. (LP-NCI)

Man Years : (Computed for the 12 month period)

Total : 4½
Professional: 1½
Other : 3

Project Description:

1) Investigation of factors influencing development of degenerative joint disease in experimental animals and man.

2) Extending descriptive pathology of human rheumatic Disease.

Methods:

1) Biomechanical factors are being studied in relation to the development of osteoarthritis. During the past year, the specific methods used have been contact X-ray microradiography of the calcified layer and electron-microscopy of the collagen of articular cartilage.

2) Comparative studies of the lesions have been pursued in other species.

3) *In vitro* culture of articular chondrocytes according to the method of A. Smith have been instituted.

Major Findings:

1) The pigment of aging cartilage described in last year's report has been partly characterized chemically. Its resistance to exhaustive proteolytic digestion permitted it to be freed from the tissue by pancreatic protease. The pigment is dialyzable and associated with the non-collagenous protein fraction.

2) Spinal ankylosis was observed in 2 of 5 Macaca monkeys between 16 and 35 years of age. One of the lesions closely resembled Marie-Strümpell disease, a condition not known to occur in species other than man. The other was of a type reported at times in various species. The interpretation is offered that it corresponds to the recently recognized human disorder, hyperostotic spondylosis.

3) Articular cartilage is attached to bone through a zone of calcification and the precise mode of this attachment would seem to be an important physical property of joint tissue. Microradiographic studies -- apparently the first of this structure -- disclose the calcified layer in man to be more densely mineralized than the subchondral bone. Several species have been studied and the findings vary among them. Experiments designed to test the reactivity of the calcified zone have been instituted.

4) The anatomic findings in previous projects have been extended.

   A. Histologic studies performed between March 24, 1967 and April 26, 1968, in addition to 7 necropsies on patients with rheumatic disease, include

Skin	Muscle	Synovium	Bone	Other
28	17	21	11	41

   B. Among the more instructive cases studied were instances of synovial chondromatosis (2), Wegener's granulomatosis (2), nodular synovitis of the temporo-mandibular joint (1), amyloidosis and calcinosis of the synovium (1 each).

Proposed Course of the Project:

The main goal of this project over the past 15 years has been an understanding of the pathogenesis of degenerative joint disease. Its principal effort in biomechanics during the last period has clarified the significance of matrix polyanions in the elastic properties of joint cartilage. The corollary that mechanical deformations of these macromolecules may selectively alter the concentration and orientation of charged groups in their vicinity suggests that joint stresses may be incorporated into a feedback system for the laying down of matrix compounds whose function is to resist the stresses. The recent development of techniques to cultivate articular chondrocytes in vitro will be applied for testing this hypothesis. The method offers promise of a new chapter in the study of repair of articular cartilage and in its metabolism.

The fine structure studies of the collagen of cartilage are making progress although this has gone slower than we had hoped. The X-ray micrographic studies of the calcified layer of articular cartilage will require 1-2 years for completion.

Publications:

1. Talal, N., Sokoloff, L. and Barth, W.F.: Extra-salivary lymphoid abnormalities in Sjögren's syndrome (reticulum cell sarcoma, "pseudolymphoma", macroglobulinemia). Amer. J. Med. 43: 50-65, July 1967.
2. Zipkin, I., Sokoloff, L. and Frazier, P.D.: A study of the effect of fluoride on bone and osteoarthritis in mice. Israel J. Med. Sci. 3: 719-725, Sept-Oct. 1967.
3. Carbone, P., Zipkin, I., Sokoloff, L., Frazier, P.D., Cook, P. and Mullins, F.: Fluoride effect on bone in plasma cell myeloma. Arch. Intern. Med. 121: 130-140, Feb. 1968.
4. van der Korst, J.K., Sokoloff, L. and Miller, E.J.: Senescent pigmentation of cartilage and degenerative joint disease. Arch. Path. (in press).
5. Sokoloff, L.: The Biology of Degenerative Joint Disease. Chicago, University of Chicago Press (in press).

Serial No. NIAMD-LEP-14
1. Experimental Pathology
2. Rheumatic Diseases
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title : Investigation of the lubrication of normal mammalian joints.

Previous Serial Number: NIAMD-LEP-15

Principal Investigator: Charles W. McCutchen, Ph.D.

Man Years: (Computed for the 12 month period)

Total : 2
Professional: 1
Other : 1

Project Description:

1) To learn why normal joints are slippery; more specifically to understand the lubricating role of the solutes in synovial fluid.

2) To measure the lubricating and rheological properties of normal and pathological human synovial fluids obtained at necropsy.

Methods:

1) The frictional properties of various pairs of mutually rubbing surfaces are measured in the presence of various lubricants. Cartilage is rubbed against cartilage, glass, rubber, etc., when lubricated with synovial fluid, .9% NaCl solution, saliva and so forth. Similarly, glass lubricated by synovial fluid and other materials is rubbed against rubber and other substances. A fruitful approach explored by Mr. Wilkins has been to use synovial mucin which has been digested with various enzymes. The rubber on glass system is used in the assay of the necropsy samples.

2) A micro Couette viscometer has been built for the assay of necropsy samples.

3) A machine has been modified to measure the friction of rabbit acetabula running against rabbit femur heads and against spheroids of varying degrees of sphericity. The object is to see if the hydrodynamic mechanism of lubrication is important under any conditions.

Major Findings:

1) A cartilage slider running against a flat glass plate enjoys poorer boundary lubrication than theory might suggest. These results are not easily related to the conditions obtained in joints, hence the rabbit joint experiments were undertaken.

2) Mr. Wilkins has obtained evidence that both the protein component and the polysaccharide of the synovial mucin are essential to its function as a boundary lubricant, the former probably being necessary for anchoring the mucin molecules to the rubbing surfaces.

3) By using multidimensional Fourier transforms between a pseudo image and a pseudo aperture, and then applying Parseval's theorem, it has been possible to derive in closed form the aperture apodization which minimizes any moment of the intensity distribution to the image.

Proposed Course of the Project:

1) To try to see why mucins are boundary lubricants while so many other long chain polymers are not. The "washability" of the adsorbed lubricant layers permits the conditions necessary for the adsorbed films to lubricate.

2) To proceed with the interferometric examination of the surface of cartilage mentioned in the last report.

3) To investigate the wear resistance of cadaveric patellar cartilage as a function of age and disease. To this end an abrading machine has been developed. Some or all of these projects will terminate when the principal investigator leaves N.I.H. in the near future.

Publication:

1. McCutchen, C.W.: Superresolution in microscopy and the Abbé resolution limit. J. Opt. Soc. Amer. 57: 1190-1192, Oct. 1967.

Serial No. NIAMD-LEP-15
1. Experimental Pathology
2. Rheumatic Diseases
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title :	Bioengineering investigation of animal joints.
Previous Serial Number:	NIAMD-LEP-13
Principal Investigator:	Frank C. Linn, A.B. in M.E.
Other Investigator :	Eric L. Radin, M.D. (Guest Worker)
Cooperating Units :	None
Man Years:	(Computed for the 12 month period)

Total : ½
Professional: ½
Other : 0

Project Description:

1) Investigation of mechanical performance *in vitro* of animal joints and their components.

2) Investigation of chemically induced changes in lubricants on the mechanical performance *in vitro* of animal joints.

Methods:

1) Lubrication and deflection characteristics of articular cartilage are studied in a specially devised testing equipment.

Major Findings:

1) The findings noted in last year's report have been extended and are presented in two papers now in press. Their mechanical performance indicates that animal joints are lubricated by several modes, principally hydrostatic and boundary. In addition to refining data concerning the roles of the protein and polysaccharide components of synovial mucin in boundary lubrication, it was observed that heparin reduces the effectiveness of this

mechanism. Synovial fluid from two cases of human rheumatoid arthritis was found to retain good lubricating ability.

Proposed Course of the Project:

This program was brought to a successful conclusion on December 31, 1967 when the principal investigator retired as planned. Several aspects of the work will continue and be incorporated into other projects of the Section on Rheumatic Diseases.

Publications:

1. Linn, F.C.: Lubrication of animal joints. I. The arthrotripsometer. J. Bone and Joint Surg. 49A: 1079-1098, Sept. 1967.
2. Linn, F.C.: Lubrication of animal joints. II. The mechanism. J. Biomech. (in press).
3. Linn, F.C. and Radin, E.L.: Lubrication of animal joints. III. The effect of certain chemical alterations of the cartilage and lubricant. Arthritis Rheum. (in press).

Serial No. NIAMD-LEP-16
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Characteristics and Function of Proteolytic Enzymes in Normal and Disease States.

Previous Serial Number: NIAMD-LEP-14

Principal Investigator: George G. Glenner, M. D.

Other Investigators: R. A. DeLellis, M. D., Staff Associate
(July 1, 1967 - July 1, 1969) and J. Sri Ram, Ph.D.

Cooperating Units: P. M. Grimley, M. D. (NCI-PA)
H. Bladen, Ph. D. (NIDR-LHP)
D. Eanes, Ph. D. (NIDR-LHP)
H. R. Keiser, M. D. (NHI-LET)
P. Cuatrecasas, M. D. (NIAMD-LEB)
V. K. Hopsu, M. D., Turku University, Finland
I. Nagatsu, M. D., Nagoya University, Japan
E. Sohar, M. D., Tel-Aviv University, Israel

Man Years: (computed for the 12 month period)

Total:	3 1/2
Professional:	2
Other:	1 1/2

Project Description:

Human Amyloid Deposits: Comparison of Two Characteristic Components

Studies on the components of human amyloid deposits has indicated that there are two morphologically characteristic components, a periodic rod which has been shown to be composed of unit structures and a non periodic fibrillar structure which has shown to be the major component of amyloid deposits. A comparison between the physical and chemical characteristics of these 2 structures has been facilitated by a purification procedure which eliminates contamination of one component by elements of the other, and the following evidence that these structures are not interconvertable has been obtained. Amino acid analysis indicates that the periodic rod is deficient in tryptophan and methionine while the fiber contains both these amino acids. Both structures have a low uronic acid composition but the periodic rod has 5% neutral sugars while the fiber 2%. Peptide mapping has shown that a tryptic digest of these two structures produces no peptides in common. After denaturation in urea or guanidine, on disc electrophoresis no protein bands are found to be common to both components. X-ray diffraction reveals an amorphous pattern for the periodic rod while the fibril has a consistent and well defined pattern showing a 4.75 Å band indicating it to be a fibrous protein of keratin type. In addition staining reactions of the two components reveal only the fibril to have Congo red birefringence under crossed polars while immunologic studies indicate that only the periodic component is antigenic. It is concluded that the periodic rod is an antigenic globular glycoprotein which does not contribute to the formation of the non-antigenic amyloid fibril, a structural glycoprotein of keratin type (in collaboration with Drs. H. Bladen, D. Eanes, and H. Keiser).

Carotid Body: Mode of Chemoreception

Surgically excised human carotid bodies have been analyzed by electron microscopy. Several ultrastructural features suggest that they are closely related to sympathetic neuroendocrine glands and autonomic ganglia: the chief cells synthesize intensely osmiophilic cytoplasmic granules, occasionally develop neuroid processes, and are intimately associated with neurilemmal sustentacular cells. Other lines of evidence indicate that the chief cell secretion is norepinephrine or a closely related bioamine-neurotransmitter. The role of chief cells in mediating chemosensation may be explained by modulated release of the bioamine in response to local metabolic conditions. Human chief cells are richly innervated. Recognizable zones of synapse display an efferent type of polarity and sometimes appear adrenergic. Thus, reflexive sympathetic excitation of chief cells may be physiologically significant in man. In terms of impulse generation, small cell units could create a miniature internal circuit for modulation of simultaneous metabolic and sympathetic efferent stimulus input. Consideration of chief cells as active couples or transducers interposed between nerve endings and the intenstitial environment in interpretation of physiologic data is suggested to replace older concepts based on a passive "sensory" mechanism (in collaboration with Dr. P. M. Grimley).

Honors: Councillor, Histochemical Society, 1966-71

Publications:

Grimley, P. M., and Glenner, G. G.: Histology and Ultrastructure of Carotid Body Paraganglioma. Comparison with the Normal Gland. Cancer 20: 1473-1488, 1967.

Grimley, P. M., and Glenner, G. G.: Ultrastructure of the Human Carotid Body. A Perspective on the Mode of Chemoreception. Circulation 37: 648-665, 1968.

Glenner, G. G.: Biography and Bibliography of Dr. Ralph D. Lillie. J. Histochem. Cytochem. 16: 3-16, 1968.

Nagatsu, I., Nagatsu, T. and Glenner, G. G.: Species Differences of Serum Amino Acid - Naphythylamidases. Enzymologia 34: 73-76, 1968.

Hopsu-Havu, V. K., Rintola, P., and Glenner, G. G.: A Hog Kidney Aminopeptidase Liberating N-terminal Dipeptides. Partial Purification and Characteristics. Acta chem. scand. 21. (in press).

Serial No. NIAMD-LEP-17
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Cytogenetics

Previous Serial Number: NIAMD-LEP-15

Principal Investigator: Dr. J. H. Tjio

Other Investigators: None

Cooperating Units: B. White, M. D. (NICHD)
W. Pahnke, M. D. (Spring Grove State Hospital)
G. Brecher, M. D. (Univ. of California, San Francisco)
T. T. Puck, M. D. (Univ. of Colorado)

Man Years: (computed for the 12 month period)

Total:	3
Professional:	1
Other:	2

Project Description:

1. Cytogenetic studies on a mouse translocation.

Cytogenetic studies in collaboration with Dr. White were carried out on a mouse translocation with reduction of chromosome number but constant number of arms. The animals were accidentally found in a colony of albino mice which had been randomly bred at the NICHD for over 3 years without any known mutagens. It appeared to be the result of centric fusion of the smallest autosome and one of the larger autosomes of the complement. Chromosome number of heterozygotes was 39 and that of homozygotes 38. The animals with 38 and 39 chromosomes were phenotypically normal with the exception of one female runt found in a litter from a mating between homozygotes. Studies of meiosis of male heterozygotes showed an autosomal trivalent at metaphase I and a low incidence of unbalanced metaphase II cells. The homologous translocation submetacentrics formed a ring bivalent at diakinesis in male homozygotes, and metaphase II cells of these animals were balanced. Progeny from matings between heterozygotes had balanced complements (N. F. = 40) of 38, 39, or 40 chromosomes in a near --1:2:1 ratio. The homozygotes and their non-translocation-bearing littermates demonstrated normal fertility, and fertility of heterozygotes was moderately reduced.

2. Cytogenetic studies on the effect of LSD.

Our interest in this work was stimulated by conflicting reports of the adverse effects of LSD on the chromosomes of human leucocytes *in vitro* and *in vivo*, and on the embryos of rats, mice, and hamsters. A pilot *in vitro* experiment with human blood cultures was performed with LSD in the final concentrations: 10 mg/cc, 1 mg/cc, 0.1 mg/cc, 0.01 mg/cc, and 0.001 mg/cc were added to the cultures at 0, 6, 24, and 68 hours after starting the cultures. We found up to ± 80% of the metaphases with chromosomal alterations in all the cultures that received LSD at 0 hour, independent of the dosage. However, in all the cultures receiving LSD at 6, 24, and 68 hours, this frequency drops to ± 15 % while the controls had only ± 10%. The cells are apparently most sensitive to the effects of LSD long before the S period of the mitotic cycle. (At 6 hours the protein synthesis is more or less over, at 24 hours the DNA synthesis period is starting and at 68 hours, the DNA synthesis is almost finished). Several studies are contemplated to confirm and extend these observations when the LSD becomes available.

(a) *In vitro* experiments on human leucocyte cultures with LSD. Series of experiments with various dosages and timing will be carried out to investigate how and when LSD affects the cell kinetics, and what the lethal dose and lowest threshold for the radiomimetic effects are for leucocytes grown *in vitro*.

(b) *In vivo* experiments on the effect of LSD on mitotic and meiotic chromosomes of laboratory animals such as rat, mouse, and chinese hamster. Series of experiments with various dosages and timing will be carried out to study the effects of the drug on the chromosomes of somatic and germ cells (spleen, bone marrow, and gonads).

(c) Cytogenetic studies are being carried out on the chromosomes of leucocyte cultures from the Spring Grove State Hospital from a series of patients before, during and after therapy. Thus far, we have studied about 100 blood samples of which only 5 have shown considerable chromosome damage.

3. Studies on radiation and drug induced chromosomal aberration.

There is no way to assess possible chromosomal damage from radioactively labeled thymidine and similar compounds which are incorporated into DNA. Theoretically, we can calculate the average exposure of the chromosomal material in terms of rads. In effect, however, we cannot readily distinguish damage due to radiation and damage due to its own decay. The matter has recently been further complicated by the discovery that the rate of chromosomal aberration depends on the position of the hydrogen atoms replaced by tritium in the thymidine molecule. At the present time it is not possible to predict chromosomal damage from computing exposure in rads, but actual measurement of the integrated biologic effect must be made. It may be possible to compare damage induced by whole body radiation of a given intensity with the effect of ingested or injected thymidine of a particular specific activity by measuring chromosomal aberration in one of the sensitive tissues in vivo such as regenerating liver or spleen. Similar problems exist in terms of drug induced chromosomal aberrations. Experiments in this direction are being carried out in collaboration with Dr. Brecher of the San Francisco Med. Center, University of California.

4. The long term growth of euploid mammalian cells in tissue culture.

This collaborative study with Dr. Puck, Dept. of Biophysics, University of Colorado, has been continued (see report of 1967).

5. Comparative chromosome studies in fish as related to phylogenetic development of the karyotypes in lower vertebrates.

Cytogenetic findings have given indications that so far as the higher animals are concerned, evolutionary changes may be related to karyological variations. The phylum Pisces is an important group of animals in the evolutionary development of vertebrates, especially the primitive groups of chordates. For an understanding of the evolutional mechanism of fish and their sex chromosome mechanism in relation to the taxonomy and the evolution of primitive vertebrates, comparative karyological studies are being carried out by Mrs. G. Johnson on spp. of the lower vertebrate families of Petromyzontidae and Polyondontidae.

Publications:

Whang-Peng, J., Tjio, J. H., and Cason, J. C.: Re-utilization of presumably degraded radioactive label from a chromosomal fraction of homologous and heterologous cells. Proc. Soc. for Exp. Biol. and Med. 125: 260, 1967.

Duvall, C. P., Carbone, P. P., Bell, W. R., Whang, Jacqueline, Tjio, J. H., and Perry, Seymour: Chronic myelocytic leukemia with two Philadelphia chromosomes and prominent peripheral lymphadenopathy. Blood 29: No. 4, Part II, 1967.

White, B. J. and Tjio, J. H.: A mouse translocation eith 38 and 39 chromosomes but normal N. F. Hereditas 58: 284-296, 1968.

Whang-Peng, J., Canellos, G., Carbone, P., and Tjio, J. H.: Clinical Implications of Cytogenetic Variants in Chronic Myelocytic Leukemia (CML). Blood. (in press).

Serial No. NIAMD-LEP-18
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolic Changes in Experimental Pathologic States

Previous Serial Number: NIAMD-LEP-16

Principal Investigator: Maria Spatz, M. D.

Other Investigators: Dr. G. L. Laqueur, Dr. M. Valsamis, Dr. R. K. Haddad

Cooperating Units: Dr. G. L. Laqueur, (LEP-NIAMD)
Dr. Marius Valsamis (Dir. of Lab., Brooklyn State Hospital, 681 Clarkson Avenue, Brooklyn, N. Y.)
Dr. R. K. Haddad (N. J. Bureau of Research in Neurology & Psychiatry, Princeton, N. J.)

Man Years (computed for the 12 month period)

Total:	2 1/2
Professional:	1
Other:	1 1/2

Project Description:

I. Hydrolysis of Cycasin by β-D-glucosidase in Skin of Newborn Rats

A. Previous studies in laboratory animals showed that cycasin was hepatoxic and carcinogenic if hydrolysis to its aglycone, methylazoxymethanol (MAM) occurred. In feeding experiments, hydrolysis was dependent on a β-D-glucosidase of bacterial origin. Cycasin when injected subcutaneously was carcinogenic only during the early postnatal period of life. These findings raised the question whether the skin of newborn rats contained a β-D-glucosidase responsible for the deglucosylation of cycasin to its aglycone.

The determination of the skin β-D-glucosidase were carried out in conventional Sprague-Dawley and Fischer rats and in germ free Sprague-Dawley rats ranging in age from the 15th prenatal to the 30th postnatal day. The following three assays were used.

1) The enzymic release of p-or-o-nitrophenol from p-or-o-nitrophenol-β-D glucosidase. 2) Indirect hydrolysis of cycasin as measured by observing the rate of glucose formation from cycasin by coupling the skin glucosidase activity to TPN reduction by means of hexokinase and

glucose-6-phosphate dehydrogenase. 3) Direct hydrolysis of cycasin to its aglycone, MAM, as measured by the rate of increase in ultraviolet absorption at 215 mμ.

β-D-glucosidase in the crude skin homogenate had the same properties in the two strains of rats and no significant difference in the enzymic activity was noted between germfree and conventional animals. The specific activity of β-D-glucosidase was related, however, to the age of the rats, being highest in the prenatal and early postnatal days. These findings were substantiated by the rate of TPN reduction of the coupled system during corresponding days when cycasin was used as substrate. The low activity of the enzyme at weaning time might not necessarily indicate disappearance of the enzyme and the possibility exists that the enzyme may only alter its properties in adult life.

Enzyme kinetics, when the amount of crude skin homogenate was varied in the reaction mixture, the amount of p-and-o-nitrophenol release from p-and-o-nitrophenyl-β-D-glucoside at optimum pH of 4.2 after 90 minutes incubation was directly proportional to the amount of homogenate added. The Km for PNPG was 2.5×10^{-5} M. The Km for cycasin was 3.3×10^{-5} M at pH 5.9 M as calculated graphically by the method of Lineweaver and Burk as modified by Dixon.

When the rate of formation of MAM from cycasin was measured, MAM was found consistently from 1-24 days of postnatal life, and the relationship between the amount of homogenate used and the percent of MAM obtained was linear.

Cycasin hydrolysis to MAM was detectable after 90 min. incubation and the release of MAM was 66% at pH 4.2; 47% at pH 5.2 and 11.8% at pH 7.0.

These observations suggests that the same skin enzyme responsible for release of p-and-o-nitrophenol from p-and-o-nitrophenyl-β-D-glucoside is responsible for the deglucosylation of cycasin. However, further studies with a soluble and purified enzyme should clarify whether this is true.

In summary the demonstration of an enzyme capable of hydrolyzing synthetic substrates such as p-and-o-nitrophenyl glucosides and the naturally occurring glucoside, cycasin, using skin as the enzyme source indicates this to be the site for cycasin deglucosylation and its conversion to the active aglycone in subcutaneously injected newborn rats. Its broader physiologic significance is still unknown. Most probably this "short-term activity" enzyme is only one of several enzymes present during tissue maturation.

B. The solubilization and purifications of the skin β-D-glucosidase is under investigation.

II. Carcinogenic Effect of Methylazoxymethanol MAM in Hamsters

A. The teratogenic effect of methylazoxymethanol MAM was demonstrated in hamsters, when MAM was injected in doses of 20 - 25 mg/kg of body weight on the 8th day of gestation (Spatz, M., Dougherty, W. J., Smith, D. W. E., Proc. Soc. Exp. Biol. Med. 124 (2), 1967.

This study describes the results of experiments in which a single intravenous administration of MAM was tested for possible carcinogenic effects in hamsters.

a) Thirty-one hamsters received a single i.v. injection of MAM 20 mg/kg body weight. At the time of injection 12 animals (7 females & 5 males) were three weeks, whereas the remaining 19 hamsters (10 females & 9 males) were 6 weeks old. b) An additional nine 6 weeks old female hamsters received 30 mg/kg of MAM intravenously. c) A total of 23 hamsters (11 females and 12 males) served as controls, and 6 of these animals received a single intravenous injection of physiologic saline at the age of three weeks.

The pathologic lesions found in the experimental animals were confined to liver, intestinal tract and gallbladder. All of the MAM injected hamsters irespective of sex, age of injection and the administrated dose of MAM displayed a severe alteration of the liver parenchyma. The most common findings listed in decreasing order of incidence were as follows: single or multiple cystadenomas, nodular hyperplasia, focal bile duct hyperplasia, hemangiomas and biliary cirrhosis. In addition one adenocarcinoma and malignant hepatoma with widespread mestastasis was found 10 and 11 months following injection of MAM in two animals. Thirty-one out of 36 hamsters surviving 7 - 13 months after MAM administration developed large intestinal lesions. 6 out of 31 animals showed focal adenomatous hyperplasia of colonic mucosa; each of the remaining 25 hamsters revealed 1 - 5 adenomas of the colon and 15 of these animals showed also 1 - 6 colonic (including cecum) carcinomas with and without metastasis. The histologic patterns of the liver and intestinal lesions in hamsters were similar to the one described previously in rats (Laqueur, et al, Virchow, Arch. Path. Anat. 340: 151, 1965 and J. Nat. Cancer Inst. 39: 355, 1967). In addition 5 of these MAM treated animals revealed hyperplasia of the gallbladder mucosa. The control animals were free of pathologic lesions.

The results of this experiment had clearly established MAM given as a single injection intravenously to have a carcinogenic effect in hamsters.

B. In previous studies with methylazoxymethanol, MAM, the proximate carcinogen of cycasin a large number of small intestinal tumors was found following repeated intraperitoneal injections of MAM to rats. These observations as well as the findings of mucosal hyperplasia of gallbladder in guinea pigs following feeding of crude cycad meal suggested a possible hepato-biliary metabolic pathway of MAM.

Therefore the investigation of the MAM biologic effects was extended to one of the small laboratory animals with a gallbladder, namely the hamsters. Five 8 week old male hamsters received three intraperitoneal injections of 20 mg/kg MAM at a three week interval.

In this experiment only 2 out of the 5 MAM injected hamsters survived 8 months after the first injection. Both of these animals developed adenocarcinoma of the gallbladder with extension to the liver and multiple colonic carcinomas.

The induction of gallbladder carcinoma supports a possible biliary pathway of MAM or its metabolites but requires further study to prove this hypothesis. So far, these observations are being used to develop a model system for such an investigation (with Laqueur).

III. Chemical Induction of Microencephaly in Rats (with Laqueur, also with Valsamis & Haddad)

Publications:

Spatz, M. and Laqueur, G. L.: Evidence of Transplacental Passage of the Natural Carcinogen Cycasin and its Aglycone. Proc. Soc. Exp. Biol. Med. 127: 201, 1968.

Hirono I., Laqueur, G. L., and Spatz, M.: Tumor Induction in Fisher and Osborne-Mendel Rats by Single Administration of Cycasin. J. Nat. Cancer Inst., May, 1968.

Hirono, I., Laqueur, G. L., and Spatz, M.: Transplantability of Cycasin-Induced Tumors in Rats with Emphysis or Nephroblastomas. J. Nat. Cancer Inst., May, 1968.

Spatz, M.: Hydrolysis of β-D-Glucosidase in Skin of Newborn Rats. Proc. Soc. Exp. Biol. Med. (in press).

Serial No. NIAMD-LEP-19
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Protein-Protein Interactions and Immune Processes in Normal and Disease States.

Previous Serial Number: NIAMD-LEP-17

Principal Investigator: J. Sri Ram, Ph. D.

Other Investigators: R. A. DeLellis, M. D., and G. G. Glenner, M. D.

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total:	3
Professional:	1
Other:	2

Project Description:

Immunological Approaches to Experimental Amyloidosis

Lack of a satisfactory method for inducing amyloidosis in laboratory animals has been a major factor in our poor understanding of the pathogenesis of amyloidosis. Studies directed at evolving a simple procedure for experimental amyloidosis revealed that amyloidosis developed in mice two weeks after the second of two biweekly intraperitoneal injections of 0.25 ml. of an emulsion made up of 25 mg. casein, 1.5 ml. complete Freund's adjuvant (F. A.) and 1.5 ml. buffered saline (PBS). Incorporation of additional M. butyricum, without casein in F. A. reduced the time required for the induction of amyloid. A single i.p. injection of 0.25 ml. of an emulsion of 25 mg. M. butyricum, 1 ml. PBS and 1 ml. complete F. A. resulted in amyloidosis in mice in two weeks. This procedure for experimental amyloidosis involving a single injection facilitated studies on the sequential cellular changes associated with the formation of amyloid, and attempts at modification of the disease with immunosuppressive procedures, such as the administration of cyclophosphamide (CPP). Administration of CPP, at a dose of 255 mg/kg, concurrently with or 1 week prior to the adjuvant, resulted in a marked decrease in the severity of the disease and a slight decrease in incidence. Even at a dose of 350 mg/kg, total suppression of amyloidosis was not observed. When CPP was administered (255 mg/kg) one week after the adjuvant, the severity of amyloidosis apparently increased.

Immunochemical Studies with Human Amyloid

Immunochemical studies were carried out with human amyloidotic spleen and liver in attempts to obtain a better understanding of the nature of amyloid. Rabbit antisera obtained by prolonged immunization, after suitable absorption with normal tissue antigens, did not react with amyloid preparations purified by fractional centrifugation or proteolytic degradation. They reacted with a protein present in serum and in amyloid deposits, possibly by absorption from the former. These studies suggest that amyloid like the other members of the fibrous protein family is either an extremely poor antigen or non-immunogenic.

Honors: J. Sri Ram was elected to the membership of the American Society for Experimental Pathology.

Publications:

Ram, J. Sri, Glenner, G. G., and DeLellis, R. A.: Amyloid I. Use of Freund's Adjuvant in Experimental Amyloidosis. Proc. Soc. Exp. Biol. Med. 127: March, 1968.

Ram, J. Sri, DeLellis, R. A., and Glenner, G. G.: Amyloid III. A Method for Rapid Induction of Amyloidosis in Mice. International Arch. Allergy and Applied Immunol. (in press).

Ram, J. Sri, DeLellis, R. A., and Glenner, G. G.: Amyloid IV. Is Human Amyloid Immunogenic? International Arch. Allergy and Applied Immunol. (in press).

ANNUAL REPORT SUMMARY

LABORATORY OF CHEMICAL BIOLOGY

This laboratory is concerned with a number of broad aspects of the chemistry and biology of macromolecules. The individual projects listed below concern themselves with structure-function relationships in proteins, the relationships between genetic information and protein structure and synthesis, the biochemistry of aggregating protein systems, and the role of many of these basic phenomena in the integrated biology of organisms; including bacteria, protozoa, and vertebrates.

I. Studies on the structure-function relationships of a nuclease from Staphylococcus aureus

Chemical structure and the effect of ligands on conformation

Staphylococcal nuclease has become an important object for biological research. The absence of disulfide bridges, the relatively low molecular weight and its enzymological properties make it of particular value for understanding the relationship between its amino acid sequence, conformation and its enzymological properties. Thus, nuclease has become subject to intensive study by a group of workers in this laboratory. Because of its scarcity and high price, a decision was made to develop a new method for the isolation of large quantities of nuclease. A reproducible method has been developed, involving adsorption on phosphocellulose, which makes possible isolation of relatively large quantities of nuclease. The application of this method on laboratory or commercial scale should considerably reduce the price of this protein. A detailed description of this method is being prepared for publication (Moravek)

The amino acid sequence deduced for the V-8 strain of Staph aureus has now been reexamined on the enzyme derived from the Foggi strain. Each of the major proteolytic fragments has been characterized as to amino acid analysis and partial sequence. The fragments have been aligned in their probable order. Most of the tryptic peptides have been sequenced and many of them are aligned in their proper arrangement within the fragments. One major amino acid change has been found so far between V-8 and Foggi: leucine 124(V-8) is replaced by histidine (Foggi) without any detectable change in enzymatic properties. Moreover, a number of differences in amide group positions have been noted between the two strains. (Taniuchi, Cusumano)

A new active derivative (nuclease-T) composed of two noncovalently-bonded fragments has been discovered. The steps in the formation of nuclease-T by trypsin in the presence of the ligands thymidine diphosphate and calcium are as follows: trypsin cleaves the bond between residues 5 and 6 very rapidly forming two fragments, nuclease-T-P_1 (residues 1-5) and nuclease-T-P_{144} (residues 6-149). The latter possesses full enzymic activity and shows helical content and heat stability similar to those of intact nuclease. Subsequent tryptic cleavage of nuclease-T-P_{144}, under the same conditions, produces nuclease-T composed of nuclease-T-P_2 (residues 6-48) and nuclease-T-P_{3a} (residues 49-149) or nuclease-T-P_{3b} (50-149). These components obtained from both nuclease-V8 and nuclease-Foggi are exchangeable to regenerate the enzymic activity. The restoration of helix upon association of nuclease-T-P$_2$ and -P$_3$ is also observed. Either of two componants alone shows no significant helix by optical rotatory dispersion and circular dichroism. (Taniuchi)

Previous studies from this laboratory had indicated that the enzymatic hydrolysis of DNA and RNA was competitively inhibited by certain monophosphonucleosides. The latter bind, in the presence of Ca^{++}, in a 1:1 molar ratio to the active site of the enzyme. This binding process appeared to be accompanied by marked changes in the environment of tyrosyl residues. The principal aim of these studies was to obtain chemical confirmation of tyrosyl-nucleotide interaction at the active site of the enzyme. The environment of about 3 tyrosines appears to be altered in the binding of pdTp and Ca^{++} to the enzyme. This was established by changes in spectrophotometric titrations, neutral solvent perturbation, ultraviolet absorption, and fluorescence properties. Reaction of nuclease with N-acetylimidazole destroys activity and O-acetylates (reversibly) 5 of the 7 tyrosines. Three of these can be protected by nucleotide and there is concomitant protection of enzyme activity. Reaction of nuclease with a large excess of tetranitromethane destroys activity and results in nitration of 5 tyrosines. With a low excess of the reagent only one tyrosine reacts and activity is lost; this was identified in the primary structure as tyrosine 85. In the presence of pdTp tyrosine 85 does not react, but instead another residue is selectively nitrated; this was identified as tyrosine 115. The two mononitrotyrosyl derivatives were purified. Certain physical properties suggested that in the native enzyme tyrosines 85 and 115 are stereochemical neighbors, and that there is some form of interaction or cooperativity between these two residues at the active site. THe mononitrotyrosyl derivatives were reduced to the corresponding aminotyrosyl derivatives. These were then treated with bifunctional reagents (difluorodinitro benzene and difluorodinitro diphenyl sulfone) at pH 5, where the aminotyrosyl residues but not other protein amino groups react. The excess reagent was then removed, and the pH raised to 9.4 to allow reaction of the other end of the biheaded reagent. The crosslinking sites were then determined.

Various low molecular weight substrates have been tested. The hydrolytic patterns toward these substrates allow description of the catalytic specificity. Kinetics have been obtained which permit resolution and control of the various steps of hydrolysis, i.e., binding, formation of enzyme-bound intermediates, and breakdown of the latter to products. The effect on these processes of Ca^{++}, nucleophiles, pH, and salts are being determined. These studies hopefully will aid in designing the optimum conditions for active site labelling, determining ionizable protein residues involved in substrate binding and hydrolysis, and ascertaining the role of Ca^{++} as an activating cation. Preliminary experiments indicate that a transient, moderately stable, enzyme substrate intermediate can be obtained by rapidly denaturing the enzyme in the presence of high concentrations of substrate. There is some evidence that hydroxylamine may trap such an intermediate.(Fuchs, Cuatrecasas, Wilchek, Schlaff)

Organic synthesis of staphylococcal nuclease and some of its fragments

Because of the nature of its sequence and of the very large amount of information available on the chemical nature of the enzymically active site, studies have been initiated toward the synthesis of the nuclease by organic chemical methods. These studies should ultimately lead to a clear understanding of the mechanism of action in molecular terms and to accumulation of valuable information on the nature of interactions between amino acid side chains that determine and stabilize the three dimensional conformation of the enzyme. Because of the length of the polypeptide chain (149 residues) classical methods of peptide synthesis do not seem to offer a particularly hopeful approach. We have, therefore, studied the applicability of the solid phase method and have examined, by a number of control studies, means to improve yields and general dependability of the method. Protected fragments with N-terminal aspartyl or glutamyl residues have been prepared by reacting hydroxysuccinimide esters of α-tBOC, ω-benzyl Asp (Glu) with the peptide cleaved from the insoluble resin, producing a new peptide with α-amino and side-chain carboxyl groups protected. A number of peptides have been highly purified by counter-current distribution. Dipeptides, tripeptides, and tetrapeptides of various composition have been coupled with nearly 100% completeness to peptides of 2-5 residues on the insoluble support. With two different coupling reagents, racemization has been prevented entirely, as tested by sterospecific enzymatic digestion. These experiments are probably the first in which peptides have been coupled without racemization with solid-phase methods.

The solid phase method has been applied to the synthesis of the smaller of the two fragments composing nuclease-T (see above). Blocked amino acids are coupled in stepwise fashion to the insoluble polystyrene resin by an adaptation of the method of Merrifield. Cleavage of the completed polypeptide chain from the resin is accomplished by anhydrous HF, with concurrent removal of the majority of blocking groups. Final removal of the ϵ-trifluoracetyl groups of lysine is by aqueous piperidine. After initial purification on Sephadex G-25, the crude polypeptide was subjected to appropriate chemical and biological tests. A polypeptide product with amino acid content resembling the native P_2 has been synthesized. The product has lysine as its only end group and migrates on disc gel and paper electrophoresis in similar fashion to its native counterpart. It is totally hydrolyzed by a mixture of proteolytic enzymes. The synthetic peptide strongly inhibits the precipitin reaction between native nuclease and its specific antibody. The crude product has a low but definite ability to generate nuclease activity when added in excess to native fragment P_3.

Although classical methods do not seem applicable to the full sequence of the nuclease, synthesis of the relatively short P_2 fragment does seem feasible. This would be the first synthesis of a material having enzymatic activity with a readily measurable assay; namely, the hydrolysis of DNA and RNA. Most of the fragments necessary for coupling have now been synthesized in sufficient quantities. Preliminary work is being performed in investigating the optimal conditions for coupling large peptide fragments.(Ontjes, Wilchek, Omenn, Zeiger, Ohno)

Related studies on the crystallography, genetics, immunology and purification of staphylococcal nuclease

The cooperating unit at MIT has prepared several heavy atom derivatives of nuclease crystals that are isomorphous with the native crystals. The use of phases from one crystal form in the Fourier analysis of other forms has indicated the correctness of the phase selections. The various derivatives are sufficiently isomorphous that data are now being collected that will permit three-dimensional Fourier synthesis of the structure at a level of 4 Å resolution. Several derivatives of nuclease lacking amino acid residues at the amino terminal end of the chain have been supplied to the MIT group, and it is hoped that these partially degraded structures which crystalize in an isomorphous form will be of help to the MIT group in locating the spatial location of the amino terminal end of the molecule. Collection of X-ray data and the preparation of further isomorphous derivatives will permit structural analysis to 2 Å resolution as judged by the excellence of the present data at lower resolutions. Variations in crystal structure will be studied as a function of the addition of certain competitive inhibitors to the native molecule.(Anfinsen, MIT)

As a preliminary to the induction and isolation of structural mutants of the nuclease, an effective and convenient medium for growth of colonies and test of nuclease production while maintaining viability has been developed: This method obviates need for replica plating, an essential part of alternate (usual) method with DNase Test Agar. Forty suspected mutants have been isolated, grown in dense culture, and assayed to confirm markedly reduced nuclease production. However, each of these has failed to give a positive immunoassay in the gel diffusion test. Thus, either regulatory mutants (less enzyme produced) or major deletions or frameshifts in the structural gene must have been produced. The desired mutant with reduced activity, but normal immunoreactivity has not yet been isolated. The isolation of specific structural mutants of this enzyme, for which amino acid sequence is known, will afford direct studies of the relationship between amino acid sequence and function of the enzyme. Since the enzyme can be hydrolyzed to two large fragments with residual activity (nuclease-T), similar experiments with mutants having alterations in structure in one or the other of the two fragments causing inactivity may produce fragments which can be combined to regenerate activity. In such an experiment the "normal" pieces of each pair might combine in a sort of molecular complementation phenomenon. (Omenn)

The reaction between rabbit antinuclease γ-globulin and various derivatives of nuclease has been studied by precipitin test and enzymatic assay. A number of chemically modified nucleases, as well as several peptide fragments of nuclease have been tested for interactions in these systems. Antinuclease γ-globulin causes complete enzymatic inactivation of nuclease, whether it forms a precipitate or a soluble antigen-antibody complex. Calcium ions and the substrate analog, thymidine-3',5'-diphosphate (Tdp) cause an inhibition of the precipitin reaction and protect nuclease from inactivation by its antibody. Ca^{++} alone, but not Tdp alone, has an inhibitory effect on the initial rate of precipitin formation. These small molecules are known to bind strongly to nuclease and to produce changes in spectral properties and in susceptibility of the enzyme to proteases. It is apparent that they also render the enzyme unrecognizable to its antibody. A mixture of the 5 cyanogen-bromide peptides of nuclease produces strong inhibition of the nuclease-antinuclease reaction while the mixed tryptic peptides have very weak inhibitory activity. A synthetic peptide comprising residues 6 through 47 is a strong inhibitor. A number of chemically modified nucleases precipitate with antibody in a fashion similar to the native protein. The reaction between antibody and the enzymatically active nitro-tyrosine 115 nuclease is inhibited by Ca^{++} and Tdp, while the reaction between antibody and enzymatically inactive nitro-tyrosine 85 nuclease is not. (Fuchs, Ontjes, Cuatrecasas)

Synthetic polypeptides prepared by the solid phase synthetic method have been found to be highly contaminated with a large number of related structures. As one method for the purification of such complex mixtures, filtration through columns prepared from immobilized macromolecules having specific affinities for the polypeptides in question has been investigated. Experiments have been initiated with the nuclease fragment P_3 attached to Sepharose columns for the purification of synthetic P_2 prepared by the solid phase method. These studies have not progressed far enough to give clearcut results. (Kato)

II. Factors determining the conformation of proteins in solution

These studies continue earlier work on the forces directing and stabilizing the tertiary structure of globular proteins. Previous studies have shown the presence of an enzyme in rat and beef liver microsomes that catalyzes the reactivation of a number of reduced or randomly cross-linked proteins. This enzyme has now been used to investigate the stability of the structure of RNase-S-protein. This material represents half of the two-component derivative of ribonuclease, RNase-S. One component, a 20-residue peptide, is bonded noncovalently to the other component, which contains 104 residues and all four SS bonds of the native RNase. The enzymic catalysis of disulfide interchange causes rapid "scrambling" of the SS bonds of S-protein. Upon addition of S-peptide to this scrambled system, a second wave of rearrangement occurs that re-establishes the original native pairing of SH groups and reconstitutes a fully active substance. These experiments prove that the structure of S-protein is not predicted by its sequence and that essential information for determining three-dimensional structure must be contributed by the S-peptide moiety. The role of ligands in modifying the amino acid sequence-determinied conformation of proteins has become more fully appreciated in the last few years. In proteins with subunits, the interaction of the separate polypeptide chains also refines the structural and functional properties of the molecules. We have continued and extended our investigations into the factors determining conformation of proteins in solution by studying the physical and chemical properties of myoglobin and malic dehydrogenase. Our study of the reversible denaturation of the chicken heart malic dehydrogenase isoenzymes has led to the conclusion that interconversion of the electrophoretically different bands does not occur under conditions of random coil behavior, when conformational heterogeneity would be eliminated. However, there is a decreasing efficiency of enzyme recovery from cathodal to anodal components. These results, in conjunction with other experiments, indicate that there are covalent differences among these isoenzymes and that the conformer hypothesis is not an adequate explanation of this system. The studies of the urea- and guanidine hydrochloride-induced denaturation of metmyoglobin and apo-myoglobin have been collated and analyzed in terms of the molecular model and the thermodynamics of denaturation. The data demonstrate a one-step denaturation process under all conditions studied, and are consistent with Tanford's model of the denaturation process. Indeed, this analysis leads to a value of 15 Kcal/mole of stabilization free energy for the heme binding, in accordance with other measurements. Kinetic measurements of the recostitution process continue to point to the existence of a special stable intermediate(heme bound to the denatured protein). Measurements of the quantum yield of tryptophan fluorescence of metmyoglobin in concentrated denaturant and the ultracentrifugation properties in denaturant, however, indicate that such a fraction must compose less than 5% of the total. It has been found that the neutral ligand Ca^{++} is required for the full reoxidation of reduced Taka amylase. This protein normally contains 4 disulfide bonds, and full reoxidation under optimal conditions in the presence of Ca^{++} results in completely native-like enzyme with full enzymic activity and normally paired half cystine residues. In the absence of Ca^{++}, only 3 of the 4 disulfide bonds in the enzymically inactive form are formed, but these have normal half cystine pairing. The subsequent addition of Ca^{++} leads to the oxidation of the last disulfide bond and the appearance of enzymic activity. The study of the effect of heme on the conformational stability of myoglobin is now under further investigation by the use of hydrogen exchange measurements. The estimation of the size of different exchangeable hydrogen pools is under investigation using tritiated water and the Sephadex filtration technique of Englander.(Kato, Ontjes, Moravek, Schechter)

III. Studies on the enzymes determined by the histidine operon in *Salmonella typhimurium* and on the regulation of their synthesis

In order to gain some insight into the mechanism of control of a polycistronic operon *in vivo*, the initial kinetics of derepression of the histidine system have been investigated. This study was done for the purpose of testing our previous hypothesis that the two modes of derepression displayed by the various histidine auxotrophs reflect different mechanisms by which translation of the messenger-RNA for the histidine operon is initiated. Various histidine auxotrophs of *Salmonella typhimurium* were grown under conditions in which histidine became a limiting growth factor in mid-log phase. The histidine operon is known to derepress when this occurs. Aliquots were withdrawn from the cultures periodically before and after derepression, and extracts prepared from these aliquots were assayed for protein and for 7 of the enzymes for histidine biosynthesis. Experiments of this type were performed on a large number of histidine auxotrophs, under conditions in which the formylating capacity of the cell had been decreased (by the addition of trimethoprim or sulfacetamide to the culture medium) or increased (by the addition of compounds known to enrich the one-carbon pool of the cell -- compounds such as adenine, serine, and methionine). The kinetics of derepression were also studied in a double mutant, blocked in both the histidine pathway and the purine pathway. Histidine auxotrophs with a block in the histidine pathway which results in the accumulation of phosphoribosylaminoimidazole carboxamide (PR-AIC) have a greatly diminished formylating capacity, and display the sequential mode of derepression; those with a block which does not result in the accumulation of PR-AIC have a normal capacity to carry out formylation reactions, and display the simultaneous mode of derepression. When the supply of formyl groups is limited, the availability of N-formylmethionyl-tRNA for polypeptide chain initiation is restricted, and translation of the histidine message is initiated at the operator end preferentially. When the supply of formyl groups is not limited, a sufficient amount of N-formylmethionyl-tRNA is available so that translation of the histidine messenger-RNA may be initiated at multiple sites. These findings provide evidence for the first time that punctuation exists, *in vivo*, in a polycistronic message, allowing certain codons to signal the initiation of translation despite their internal positions.

A study was undertaken in order to examine the question of whether our previous findings on the kinetics of derepression of the enzymes for histidine biosynthesis were of general applicability. In order to maintain the same genetic background used in the previous studies, episome from *E. coli*, carrying the lactose operon, was introduced into *Salmonella typhimurium*. It has been found that the wild type organism (which displays the sequential mode of derepression of the histidine enzymes) and *hisG52* (which displays the simultaneous mode) both display a pattern of induction of the lactose enzymes which is essentially simultaneous. However, as in the case of the histidine system, reducing the formylating capacity of the cell results in sequential induction of the lactose enzymes. The study will be continued to determine whether the two modes of induction already observed are truly dependent upon the formylating capacity of the cell.

A kinetic study of the process of repression of the enzymes for histidine biosynthesis was also undertaken to gain some insight into the mechanism of the repression process. Various histidine auxotrophs of *Salmonella typhimurium* were grown under conditions in which histidine became a limiting growth factor in mid-log phase. After one hour, excess histidine was added to the culture, and the growth rate immediately returned to the fast rate characteristic of repressed cells. Aliquots were withdrawn from the culture medium periodically during the period of slow growth due to histidine limitation and following the addition of excess histidine. These aliquots were assayed for seven of the enzymes for histidine biosynthesis. The results indicate that the first enzyme for histidine biosynthesis is involved in regulation of translation of the histidine message. What is more, this involvement is realized through the allosteric site of the enzyme. Thus, a clear interrelationship has been established between the two most important control mechanisms for curtailing the biosynthesis of metabolites in bacterial cells -- repression and feedback inhibition.(Goldberger, Kovach, Berberich, Ballesteros)

IV. Studies on aggregating protein systems

These studies have involved a variety of biological materials, including β-galactosidase from several organisms, trichocysts of paramecium, and membranes of the red blood cell. All of the systems studied involve specific aggregation of protein subunits to form either enzymically active oligomers or specific cell structures.

Various physico-chemical properties of several bacterial β-galactosidases were studied in an attempt to elucidate the basis and nature of the various forms of this polymeric enzyme. The β-galactosidase of constitutive or fully induced *E. coli* K12 is seen on disc gels as a number of isoenzymes that represent the tetramer and higher aggregates. The β-galactosidase specified by the P-lac episome from *P. mirabilis* (studied in *S. marcescens*) forms isoenzymes which are more labile than those of *E. coli* and which have a higher ratio of tetramer to the other forms in fresh extracts. The difference in isoenzymes resides in the structure of the P-lac enzyme and is not due to a deficiency in some "aggregating factor" as *S. marscescens* carrying the F-lac episome from *E. coli* forms the *E. coli* isoenzymes. The β-galactosidase synthesized by an inducible strain of *A. aerogenes* does not form isoenzymes. The *Aerobacter* β-galactosidase has a sedimentation coefficient similar to that of the *E. coli* tetramer and will hydridize with it but differs in antigenic structure and amino acid composition. The β-galactosidases of *Aerobacter* and P-lac are less stable to thermal inactivation and urea denaturation than the *E. coli* enzyme with an order of stability: E. coli > P-lac > Aerobacter. The increased sensitivities correlate with a more rapid dissociation of the tetramer to the monomer. These data suggest that the intratetrameric bonds of the isoenzymes must have their structural basis in the intermonomeric bonds of the tetramer. The multiple forms of β-galactosidase of E. coli K12 exist as polymeric aggregates of increasing size ranging from 4N, 6N, 8N, 10N, 12N, and 16N units. The heavier aggregates (6N-16N) are more labile to a variety of treatments and dissociate through 4N and 2N particles becoming monomeric units (1N). The monomeric subunits of 1N are chemically identical in all species examined. Electron microscopy of the 4N, 6N, and 8N forms has revealed tetramers and "rosette" structures consisting of 6 and 8 units. These results demonstrate that the formation of higher aggregates does not involve a simple aggregation of the predominant 4N species as previously thought, but involve possibly dimers and direct association of monomers.

Undischarged trichocysts from the pellicle of Paramecium aurelia have been isolated, and experiments have been performed to characterize the structural proteins involved in this structure and their possible functional role. Electron microscopy of these structures has revealed two clearly different regions of structure together with a striation pattern only seen previously in the discharged trichocyst.

Structural components of the human red cell membrane have also been fractionated and subjected to physical and chemical study. By subjecting isolated membranes of red blood cells to various extracting media made up of low ionic strength buffers, chelating agents, and β-mercaptoethanol, we have solubilized approximately 25% of the total membrane-bound protein. This protein appears to be homogeneous by several physical criteria, and has the capacity to polymerize into helically coiled polymers which are identical in their ultrastructural appearance to actin, a fibrous protein of muscle. It was established that this protein extracted from red cells was a specific component of the red cell membrane by showing that antibodies directed against this material did not react with serum protein or proteins released from red cells during hemolysis. It was also found that antibodies against the membrane protein did not react with purified preparation of actin extracted from the same animals. The ultracentrifuge and electrophoretic properties of the membrane protein differed from those of muscle actin. (Steers, Marchesi, Erickson, Beisson)

Serial No. NIAMD-LCB-1
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The tyrosine residues at the active site of staphylococcal nuclease

Previous Serial Number: NIAMD-LCB-3

Principal Investigators: Dr. Pedro Cuatrecasas
Dr. Sara Fuchs

Other Investigators: Dr. Christian B. Anfinsen
Dr. Sheldon Schlaff

Man Years

Total: 1 1/2
Professional: 1 1/2

Project Description:

Objectives: Previous studies from this laboratory had indicated that the enzymatic hydrolysis of DNA and RNA was competitively inhibited by certain monophosphonucleosides. The latter bind, in the presence of Ca^{++}, in a 1:1 molar ratio to the active site of the enzyme. This binding process appeared to be accompanied by marked changes in the environment of tyrosyl residues. The principal aim of these studies was to obtain chemical confirmation of tyrosyl-nucleotide interaction at the active site of the enzyme.

Methods Employed and Major Findings:

1) The environment of about 3 tyrosines appears to be altered in the binding of pdTp and Ca^{++} to the enzyme. This was established by changes in spectrophotometric titrations, neutral solvent perturbation, ultraviolet absorption, and fluorescence properties.

2) In contrast to tyrosine, the environment of the single tryptophane residue is unchanged during binding of the substrate analogue. Further, this residue appears to be inaccessible to solvent in the native enzyme.

3) Reaction of nuclease with N-acetylimidazole destroys activity and O-acetylates (reversibly) 5 of the 7 tyrosines. Three of these can be protected by nucleotide and there is concomitant protection of enzyme activity.

4) Reaction of nuclease with a large excess of tetranitromethane destroys activity and results in nitration of 5 tyrosines. With a low excess of the reagent only one tyrosine reacts and activity is lost; this was identified in the primary structure as tyrosine 85. In the presence of pdTp tyrosine 85

does not react, but instead another residue is selectively nitrated; this was identified as tyrosine 115. The two mononitrotyrosyl derivatives were purified. Certain physical properties suggested that in the native enzyme tyrosines 85 and 115 are stereochemical neighbors, and that there is some form of interaction or cooperativity between these two residues at the active site.

5) The mononitrotyrosyl derivatives were reduced to the corresponding aminotyrosyl derivatives. These were then treated with bifunctional reagents (difluorodinitro benzene and difluorodinitro diphenyl sulfone) at pH 5, where the aminotyrosyl residues but not other protein amino groups react. The excess reagent was then removed, and the pH raised to 9.4 to allow reaction of the other end of the biheaded reagent. The crosslinking sites were then determined. Tyrosine 85 was found to crosslink to tyrosine 115 and to lysine 116, whereas tyrosine 115 crosslinked to lysine 53 and lysine 136. These studies confirmed the spatial proximity of the two tyrosyl residues. Further, this represents a chemical probe for determining the folding of the polypeptide chain of the native enzyme.

6) Alkylation of nuclease with iodoacetic acid or iodoacetamide was used as a probe to determine the residues necessary for biological activity. Inactivation (up to 90%) was achieved with large excess of reagent (1000 fold) and was essentially pH and calcium independent (pH range 5.5-8.0, calcium 0-0.1 M). If alkylation was carried out in urea or at pH's where structure was affected a much faster reaction took place.

Iodoacetic acid C^{14} reacts with nuclease to a total of 10-12 residues at completion while only slightly less if protection of biological activity is attempted with pdTp. However, peptide maps on samples throughout the alkylation reaction (0-24 hrs) reveal marked qualitative differences. Three peptides are alkylated initially in both protected and unprotected samples after which differences in the maps apply (4-5 residues). These peptides will be identified and correlation made to the chemical structure and biological activity.

Significance to Biomedical Research: These studies represent the beginning of attempts to define the amino acid residues and their interactions at the active site of a catalytically active macromolecule. Such information will be essential to the understanding and description of the dynamic processes involved in catalysis. Hopefully, our general understanding of proteins may be enhanced, providing some help in the pursuit of more complicated biological problems concerned with functional and structural proteins.

Proposed Course of Project: Attempts are being made to locate other functional residues at the active site. These studies will then be correlated with studies designed to chemically map the active site region: affinity labelling, kinetics of binding and hydrolysis, isolation of enzyme-substrate intermediates. The overall aim is to ultimately provide a three-dimensional view of the structural and dynamic processes involved in catalysis.

Publications:

Cuatrecasas, P., Fuchs, S., and Anfinsen, C.B.: The binding of nucleotides and calcium to the extracellular nuclease of Staphylococcus aureus: studies by gel filtration. J. Biol. Chem. 242: 3063-3067, July, 1967.

Cuatrecasas, P., Fuchs, S., and Anfinsen, C.B.: The interaction of nucleotides with the active site of staphylococcal nuclease: spectrophotometric studies. J. Biol. Chem. 242: 4759-4767, Oct. 1967.

Fuchs, S., Cuatrecasas, P., and Anfinsen, C.B.: An improved method for the purification of staphylococcal nuclease. J. Biol. Chem. 242: 4768-4770, Oct. 1967.

Cuatrecasas, P., Edelhoch, H., and Anfinsen, C.B.: Fluorescence studies of the interaction of nucleotides with the active site of the nuclease of Staphylococcus aureus. Proc. Nat. Acad. Sci. 58: 2043-2050, Nov. 1967.

Cuatrecasas, P., Fuchs, S., and Anfinsen, C.B.: The effect of a competitive inhibitor on the acetylation of tyrosyl and lysyl residues of staphyloccal nuclease. Biochim. Biophys. Acta, in press.

Serial No. NIAMD-LCB-2
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunologic Studies of Staphylococcal Nuclease; Relationship Between Antigenic and Enzymatic Activities.

Previous Serial Number: none

Principal Investigator: Dr. Sara Fuchs

Other Investigators: Dr. Pedro Cuatrecasas, Dr. David Ontjes
Dr. Christian B. Anfinsen

Man Years

Total: 1 1/8 (professional)

Project Description:

Objectives:
1) To prepare antibodies to nuclease and to define the antigenic sites with which they react.
2) To study modifications in the primary or tertiary structure of nuclease which alter its antigenicity.
3) To study the relationships between the binding of specific antibodies and the binding of substrate and metal ions.

Methods employed: The reaction between rabbit antinuclease γ-globulin and various derivatives of nuclease has been studied by precipitin test and enzymatic assay. A number of chemically modified nucleases, as well as several peptide fragments of nuclease have been tested for interactions in these systems.

Major Findings: Antinuclease γ-globulin causes complete enzymatic inactivation of nuclease, whether it forms a precipitate or a soluble antigen-antibody complex.

Calcium ions and the substrate analog, thymidine-3',5'-diphosphate (Tdp) cause an inhibition of the precipitin reaction and protect nuclease from inactivation by its antibody. Ca^{++} alone, but not Tdp alone, has an inhibitory effect on the initial rate of precipitin formation. These small molecules are known to bind strongly to nuclease and to produce changes in spectral properties and in susceptibility of the enzyme to proteases. It is apparent that they also render the enzyme unrecognizable to its antibody.

A mixture of the 5 cyanogen bromide peptides of nuclease produces strong inhibition of the nuclease-antinuclease reaction, while the mixed tryptic peptides have very weak inhibitory activity. A synthetic peptide comprising residues 6 through 47 is a strong inhibitor. A number of chemically modified nucleases precipitate with antibody in a fashion similar to the native protein. The reaction between antibody and the enzymatically active nitro-tyrosine 115 nuclease is inhibited by Ca^{++} and Tdp, while the reaction between antibody and enzymatically inactive nitro-tyrosine 85 nuclease is not.

Significance to Biomedical Research: In these studies specific antibodies have been used largely as probes for demonstrating the presence of structural changes induced in the antigen by a variety of means. Immunochemistry will continue to be a very useful and sensitive tool for this purpose.

In addition, it is of interest to know which linear sequences of amino acids tend to be most immunogenic in a protein of known primary structure. Such information will be of even greater interest when the three dimensional structure of the molecule is known.

Proposed Course of Project:

1) Further systematic testing of native peptide fragments for their ability to interact with antibodies to native nuclease.
2) Organic synthesis of any peptides found to have antigenic properties, to determine the structural features that are important for antigenicity.
3) Use of synthetic peptides as immunogens in an attempt to produce antibodies directed at a given linear sequence in the native enzyme.

Serial No. NIAMD-LCB-3
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Organic Synthesis of Fragment P_2 by Solid Phase Methods

Previous Serial Number: NIAMD-LCB-1

Principal Investigator: Dr. David Ontjes

Other Investigator: Dr. Christian B. Anfinsen

Man Years

Total: 1 1/8
Professional: 5/8
Other: 1/2

Project Description:

Objectives:
1) To synthesize fragment P_2 (residues 6 through 47) of nuclease as a means of studying structural requirements for active complex formation with native fragment P_3.
2) To test the usefulness of the solid-phase method of peptide synthesis in synthesizing large polypeptides.

Methods Employed: Blocked amino acids are coupled in stepwise fashion to an insoluble polystyrene resin by an adaptation of the method of Merrifield. Cleavage of the completed polypeptide chain from the resin is accomplished by anhydrous HF, with concurrent removal of the majority of blocking groups. Final removal of the ϵ-trifluoroacetyl groups of lysine is by aqueous piperidine.

After initial purification on Sephadex G-25 the crude polypeptide may be subjected to appropriate chemical and biological tests. Material which shows low biological activity may be purified further by taking advantage of its ability to bind specifically to native fragment P_3. A column consisting of sepharose to which native P_3 is covalently bound is capable of binding native P_2 in an enzymatically active state. Such a column should be of value for the "functional" purification of the synthetic P_2.

Major Findings: A polypeptide product with amino acid content resembling the native P_2 has been synthesized. The product has lysine as its only end group, and migrates on disc gel and paper electrophoresis in similar fashion to its native counterpart. It is totally hydrolyzed by a mixture of proteolytic enzymes. The synthetic peptide strongly inhibits the precipitin reaction between native nuclease and its specific antibody.

The crude product has a low but definite ability to generate nuclease activity when added in excess to native fragment P_3.

Significance to Biomedical Research: Production of a semi-synthetic enzyme, one third of whose amino acid sequence is the product of organic synthesis, is an approach to the total synthesis of the enzyme. Experience gained with solid phase methods indicates that they may be of value in the synthesis of longer polypeptides.

Proposed Course of Project:

1) Further improvements in the chemical details of solid phase synthesis, including a search for a more suitable blocking group for histidine, and attempts to use milder conditions for cleavage and deblocking.

2) Further development of methods for purifying the synthetic product according to its ability to bind specifically to fragment P_3.

3) Assessment of the binding and activating properties of synthetic P_2 analogs.

Publications:

Anfinsen, C.B., Ontjes, D., Ohno, M., Corley, L. and Eastlake, A.: The synthesis of protected peptide fragments of a staphylococcal nuclease. Proc. Nat. Acad. Sci. 58: 1806-1811, Oct. 1967.

Serial No. NIAMD-LCB-4
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The synthesis of the P2 fragment of S. aureus by classical chemical methods.

Previous Serial Number: NIAMD-LCB-1

Principal Investigators: Dr. Meir Wilchek
Dr. Allen R. Zeiger

Other Investigator: Dr. Christian B. Anfinsen

Man Years

Total: 1 3/4
Professional: 1 1/4
Other: 1/2

Project Description:

Objectives: The amino acid sequence of S. aureus nuclease has been determined in this lab. The enzyme consists of 149 amino acids, 23 of which are lysine and 5 of which are arginine. In the absence of Ca^{++} and d-TDP, trypsin can cleave all of the peptide bonds of the basic amino acids. In the presence of Ca^{++} and d-TDP, however, trypsin can cleave only two bonds. This results in three peptide fragments; i.e. P_1 (aa_{1-5}), P_2 (aa_{6-49}) and P_3 (aa_{50-149}). None of these fragments contains enzymatic activity by itself. If the P_2 and P_3 fragments are mixed, however, 10% of the native nuclease activity is obtained.

Clearly, the chemical methods now available preclude the synthesis of an enzymatically active nuclease. The synthesis of the relatively short P_2 fragment does, however, seem feasible. This would be the first synthesis of a material having enzymatic activity with a readily measurable assay; namely, the hydrolysis of DNA and RNA.

Methods Employed: Any approach to the synthesis of a long polypeptide chain with biological activity must take three major obstacles into account; e.g., yield, impurity and racemization of the products.

The approach which we feel best overcomes these obstacles is that of synthesizing blocked peptide fragments ending in proline and glycine. The 44 amino acid P_2 fragment contains 4 prolines and 2 glycines.

The synthesis of such fragments and their subsequent coupling means that any amino acid would not undergo more than 14 or 15 additional steps before reaching the final product. Of course, stepwise addition could require as much as 43 such steps. The coupling of fragments probably entails less loss of material (by virtue of fewer steps) and would thus require less starting material.

The use of blocking groups as N-tert-butyloxycarbonyl, N-carbobenzoxy, N-nitro, O-benzyl, t-butyl and methyl esters decreases the possibility of side reactions and ensures purer products. In addition, the coupling of large fragments entails easier separation of unreacted reactants from the products.

The use of proline and glycine as carboxyl terminal groups for these fragments is a necessary precaution against racemization. Glycine is not optically active and proline does not have any tendency to racemize. Thus, the chemical techniques employed in coupling large fragments can be more drastic than those usually employed without fear of racemization.

The synthesis of the individual fragments will be done by adding single N-blocked amino acid to the existing peptide fragments. Reports on this technique generally have claimed little or no racemization under the usual coupling conditions.

Major Findings: Most of the fragments have now been synthesized in sufficient quantities (2 mM).

Significance to Biomedical Research: The chemical sequence of the amino acid sequence of a polypeptide having biological activity would furnish strong proof of the relationship between primary structure tertiary folding and enzymatic activity.

Proposed Course of Project: Hopefully, the synthesis of the fragments will be completed within the next couple of months. Meanwhile, preliminary work is being performed in investigating the optimal conditions for coupling large peptide fragments.

Serial No. NIAMD-LCB-5
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the synthesis of peptides by the solid phase method.

Previous Serial Number: NIAMD-LCB-1

Principal Investigators: Dr. Christian B. Anfinsen
Dr. Gilbert S. Omenn
Dr. David A. Ontjes
Dr. Meir Wilchek

Cooperating Unit: Dr. M. Ohno (NIAMD:LC)

Man Years

Total: 2
Professional: 1 1/4
Other: 3/4

Project Description:

Objectives:
1) To synthesize the nuclease by organic chemical methods;
2) To evaluate the methods of solid-phase synthesis and extend its application from small peptides to proteins the size of this enzyme.

Methods Employed:
1) Solid-phase peptide synthesis: stepwise addition of protected amino acids to a growing peptide chain on an insoluble polymeric support, following by cleavage of the peptide from the resin with HF or with HBr and by purification.
2) Purification of peptide fragments by chromatography and by countercurrent distribution. Tests for homogeneity of the product by melting-point, dansyl chloride determination of N-terminal residue, electrophoresis, chromatography, and amino acid analysis of acid-hydrolyzed and of enzymatically-digested peptide.
3) Coupling of peptide fragments to peptide on solid phase support with selected coupling reagents, evaluating completeness of reaction and preservation of stereospecificity.
4) Removal of carboxyl protecting groups by enzymatic methods.

Major Findings.

1) Synthesis on the resin and fractionation of oligopeptides of lysine, of histidine, and of arginine indicate incomplete addition of monomer at each step. Thus, synthesis of heptalysine yielded only 84% hepalysine, the remainder being shorter homologs resulting from an average of 98% coupling efficiency at each step.

2) The dansyl chloride method of determination of N-terminal residues has been applied at various steps as the peptide chain is extended, to identify incomplete addition of particularly troublesome monomers, e.g. histidine.

3) Special problems of individual amino acid residues and their protecting groups have been investigated. Carbobenzoxy is now used instead of benzyl to protect the histidine imidazole function. Oxidation of the sulfur atom of methionine has been circumvented by use of reducing agents during cleavage steps and by substitution of isosteric norleucine for methionine, both in chemical and biosynthetic experiments.

4) Protected fragments with N-terminal aspartyl or glutamyl residues have been prepared by reacting hydroxysuccinimide esters of α-tBOC, ω-benzyl Asp (Glu) with the peptide cleaved from the insoluble resin, producing a new peptide with α-amino and side-chain carboxyl groups protected. Several such peptides have been highly purified by counter-current distribution.

5) Dipeptides, tripeptides, and tetrapeptides of various composition have been coupled with nearly 100% completeness to peptides of 2-5 residues on the insoluble support. With two different coupling reagents, racemization has been prevented entirely, as tested by stereospecific enzymatic digestion. These experiments are probably the first in which peptides have been coupled without racemization with solid-phase methods.

6) Several peptide fragments within the sequence of Staph. nuclease have been prepared by stepwise coupling of amino acids to Leu-Gly-Gly-OEt. This C-terminal tripeptide moiety could then be removed by digestion with the bacterial protease, thermolysin exposing the terminal carboxy group on the desired peptide.

Significance to Biomedical Research: The chemical synthesis of an active enzyme will establish the hypothesis that the linear amino acid sequence expresses the genetic message for enzyme conformation and activity. The ability to prepare and couple protected peptide fragments will permit alteration of specific residues in a particular peptide to test directly the influence of individual amino acid substitutions or modifications on structural and functional properties of the whole enzyme molecule.

Proposed Course of Project:

1) Synthesize and purify additional protected peptide fragments.

2) Improve conditions both for solid-phase and for soluble coupling of peptides.

3) Extend coupling on solid-phase support to longer peptides and to specific peptides of the nuclease sequence.

4) Combine the various synthetic, cleavage, and coupling techniques developed and applied in this laboratory to obtain the entire nuclease structure.

Publications:

Anfinsen, C.B., Ontjes, D., Ohno, M., Corley, L., and Eastlake, A.: The synthesis of protected peptide fragments of a Staphylococcal nuclease. *Proc. Nat. Acad. Sci.* 58: 1806-1811, Oct. 1967.

Ohno, M. and Anfinsen, C.B.: Removal of protected peptides by hydrazinolysis after solid phase synthesis. *J. Am. Chem. Soc.* 89: 5994-5995, Nov. 1967.

Serial No. NIAMD-LCB-6
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1967

Project Title: Mechanisms of catalysis of staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-3

Principal Investigators: Dr. Pedro Cuatrecasas
Dr. Meir Wilchek

Other investigator: Dr. Christian B. Anfinsen

Man Years

Total: 1 1/8
Professional: 1 1/8

Project Description:

Objectives: Previous studies in this laboratory have shed some light on amino acid residues present at the active site of staphylococcal nuclease. Studies have been started designed to elucidate catalytic mechanisms by 1) determination of kinetic properties of hydrolysis of phosphodiester linkages of low molecular weight substrates, and 2) covalent labelling of substrate-like compounds to the active site of the enzyme.

Methods Employed and Major Findings:

1) Various low molecular weight substrates have been purchased and synthetized. The hydrolytic patterns toward these substrates allow description of the catalytic specificity.

2) Kinetics have been obtained which permit resolution and control of the various steps of hydrolysis, i.e., binding, formation of enzyme-bound intermediates, and breakdown of the latter to products. The effect on these processes of Ca^{++}, nucleophiles, pH, and salts are being determined. These studies hopefully will aid in: designing the optimum conditions for active site labelling, determining ionizable protein residues involved in substrate binding and hydrolysis, and ascertaining the role of Ca^{++} as an activating cation.

3) Studies are in progress to determine the possible synthetic and phosphoryl transferring functions of the enzyme.

4) Labelling of the active site with substrates and pseudosubstrates. Preliminary experiments indicate that a transient, moderately stable, enzyme-substrate intermediate can be obtained by rapidly denaturing the enzyme in

the presence of high concentrations of substrate. There is some evidence that hydroxylanine may trap such an intermediate.

5) Labelling of the active site with bifunctional "quasi substrates." Functional groups capable of irreversible reaction with protein side chains are being attached on hydrolyzable substrates. The cleavage site will hopefully transiently "fix" the substrate in position while the functional group reacts with neighboring residues.

Significance to Biomedical Research: These studies stress the correlation of the functional and dynamic properties of catalysis with the structural features of the enzyme active site. Such studies could provide better understanding of how unique structural arrangements in proteins permit very specific and highly efficient performance of biologic functions.

Proposed Course of Project: This has been incorporated in the section on Methods and Findings.

Publications:

Cuatrecasas, P., Fuchs, S., and Anfinsen, C.B.: Catalytic properties and specificity of the extracellular nuclease of *Staphylococcus aureus*. *J. Biol. Chem.* 242: 1541-1547, April 1967.

Serial No. NIAMD-LCB-7
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Isolation of structural mutants of the enzyme staphylococcal nuclease.

Principal Investigator: Dr. Gilbert S. Omenn

Man Years

Total: 1/2
Professional: 1/2

Project Description:

Objectives: To induce and isolate structural mutants of the staphylococcal enzyme nuclease; to then determine structural and functional differences and correlate these.

Methods Employed:

Strain: Foggi strain, Staphylococcus aureus, from which the nuclease has been isolated for previous studies in this laboratory of amino acid sequence, immunochemistry, conformation, and limited proteolysis.

Mutagenesis: chemical treatment with the effective mutagen N-methyl-N'-nitro-N-nitroso-guanidine; and, separately, radiation with cobalt-60.

Growth conditions for selection of mutants: agar plates containing DNA plus the fluorescent dye acridine orange -- the plate is fluorescent except around those colonies which produce extracellular nuclease, which cleaves the DNA of the DNA-acridine orange complex and causes a halo of defluorescence.

Other methods for study of selected colonies:

a) growth overnight to dense culture in medium containing Casamino Acids, salts, yeast hydrolysate;

b) assay for quantitative nuclease activity by hyperchromic effect of DNA hydrolysis (method of Cuatrecasas, Fuchs, & Anfinsen);

c) coagulase test, checking coordinate control of this other extracellular enzyme;

d) Ouchterlong gel immunodiffusion, to demonstrate immunologically reactive nuclease.

Major Findings:

1) An effective and convenient medium for growth of colonies and test of nuclease production while maintaining viability has been developed: fluorescent agar medium described above. This method obviates need for replica plating, an essential part of alternate (usual) method with DNase Test Agar.

2) 40 suspected mutants have been isolated, grown in dense culture, and assayed to confirm markedly reduced nuclease production; however, each of these has failed to give a positive immunoassay in the gel diffusion test. Thus, either regulatory mutants (less enzyme protein produced) or major deletions or frameshifts in the structural gene must have been produced. The desired mutant with reduced activity, but normal immunoreactivity has not yet been isolated.

3) Isolated colonies with reduced nuclease activity also seem to have reduced coagulase activity, suggesting a coordinated control of extracellular enzyme production and/or release.

4) Attempts to liberate intracellular nuclease activity by sonication and by French press disruption of cells have yielded none; presumably all or nearly all enzyme activity is extracellular.

Significance to Biomedical Research: The isolation of specific structural mutants of this enzyme, for which amino acid sequence is known, will afford direct studies of the relationship between amino acid sequence and function of the enzyme. Since the enzyme can be hydrolyzed to two large fragments with residual activity (nuclease-T), similar experiments with mutants having alterations in structure in one or the other of the two fragments causing inactivity may produce fragments which can be combined to regenerate activity. In such an experiment the "normal" pieces of each pair might combine in a sort of molecular complementation phenomenon.

The indication that control of extracellular enzymes in Staphylococcus aureus is coordinated may be important in unraveling the role of these potent enzymes in the pathogenesis of Staphylococcal infections.

Proposed Course of Project:

1) Continue search with techniques now adapted to screening for structural mutants with intact immunoreactivity but little or no enzymatic function.

2) Use such mutants, when found, for structure determination, for conformation studies, and for molecular complementation studies.

3) Attempt further mutation of isolated single-site mutants both to determine revertability and to seek double-site mutants with regeneration of activity.

4) Use thymidine-requiring mutant to try to devise an enrichment method for the selection of organisms lacking this enzyme.

5) Continue to correlate coagulase and nuclease activities, as well as pigment production and phage type to seek some information about the control of these characteristic functions of the pathogenic staphylococcus.

Serial No. NIAMD-LCB-8
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the microsomal enzyme that catalyzes sulfhydryl-disulfide interchange in proteins.

Previous Serial Number: NIAMD-LCB-2

Principal Investigator: Ikunoshin Kato

Other Investigator: Christian B. Anfinsen

Man Years:

Total: 1/4
Professional: 1/4

Project Description:

Objectives: Previous studies in this laboratory have shown the presence of an enzyme in rat and beef liver microsomes that catalyzes the reactivation of a number of reduced or randomly cross-linked proteins. This enzyme has now been used to investigate the stability of the structure of RNase-S-protein. This material represents half of the two-component derivative of ribonuclease, RNase-S. One component, a 20-residue peptide, is bonded noncovalently to the other component which contains 104 residues and all four SS bonds of the native RNase.

Methods Employed: The microsomal enzyme which catalyzes disulfide interchange has been added to S-protein in the presence and absence of S-peptide. Evidence for rearrangement of disulfide bonds and total structure may be obtained by assaying for activity after adding S-peptide to the treated S-protein.

Major Findings: The enzymic catalysis of disulfide interchange causes rapid "scrambling" of the SS bonds of S-protein. Upon addition of S-peptide to this scrambled system, a second wave of rearrangement occurs which re-establishes the original native pairing of SH groups and reconstitutes a fully active substance. These experiments prove that the structure of S-protein is not predicted by its sequence and that essential information for determining three-dimensional structure must be contributed by the S-peptide moiety.

Significance to Biomedical Research: This work is part of a continuing study on the mechanism of the last stages of protein biosynthesis. An understanding of this mechanism may have direct bearing on an understanding of various disease states in man, especially genetically determined diseases, in which a faulty protein may be synthesized due to the inability of the protein to fold up properly into the "correct" native form.

Proposed Course of Project: This project has now been terminated.

Publications:

Anfinsen, C. B.: The formation of the tertiary structure of proteins. In The Harvey Lectures, Series 61. New York, Academic Press, 1967, pp. 95-116.

Serial No. NIAMD-LCB-9
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The use of enzymes attached to insoluble supports in the purification of proteins and protein derivatives.

Previous Serial Number: None

Principal Investigator: Ikunoshin Kato

Other Investigator: Christian B. Anfinsen

Man Years:

Total: 3/4
Professional 3/4

Project Description:

Objectives: Synthetic polypeptides prepared by the solid phase synthetic method have been found to be highly contaminated with a large number of related structures. As one method for the purification of such complex mixtures, filtration through columns prepared from immobilized macromolecules having specific affinities for the polypeptides in question has been investigated.

Methods Employed: Ribonuclease-S consists of two noncovalently bonded fragments, S-peptide and S-protein. The latter contains 104 amino acid residues and all four disulfide bonds of the parent protein. Similarly Staph nuclease can be cleaved into two fragments, peptides P2 and P3, which also aggregate noncovalently to produce an enzymically active complex. Synthetic preparations of S-peptide and of nuclease fragment P2 (containing 43 amino acids) have been passed through columns prepared by attaching either S-protein or fragment P3 to a Sepharose support.

Major Findings:

1. An S-peptide derivative containing the first 16 amino acids of natural S-peptide and capable of regenerating full ribonuclease activity when added to S-protein has been prepared by solid phase synthesis. The crude material gives rather poor amino acid analyses and, even at high excess, is able to regenerate only 50% of potential ribonuclease activity when added to S-protein. When the crude preparation is passed through a Sepharose S-protein column (prepared by adding S-protein to Sepharose previously treated at pH 11 with CNBr by the method of Porath), a fraction binds tightly which upon subsequent elution from the column with 50% acetic acid gives nearly theoretical analyses and is capable of reactivating S-protein with an efficiency

comparable to that shown by the natural S-peptide.

2. Similar experiments have been initiated with the nuclease P3 in Sepharose column for the purification of synthetic P2 prepared by the solid phase method. These studies have not progressed far enough to give clearcut results.

Significance to Biomedical Research: These studies will add to our knowledge of the formation of active centers of enzymes and of the nature of specific interactions in protein structure.

Proposed Course of Project: The studies described above are being continued and include an investigation of optimal conditions for binding and elution.

Serial No. NIAMD-LCB-10
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Amino acid sequence of the nuclease of Staphylococcus aureus (Foggi strain).

Previous Serial Number: NIAMD-LCB-1

Principal Investigator: Dr. Charles L. Cusumano

Other Investigators: Dr. Christian B. Anfinsen
Dr. Hiroshi Taniuchi

Man Years

Total: 1 3/4
Professional: 1 1/4
Other: 1/2

Project Description:

Objectives: To determine the amino acid sequence of Foggi strain nuclease.

Methods Employed and Major Findings:

1) The nuclease chain is cut by chemical means into several large fragments which are then purified. Each of these fragments is then cleaved into a number of peptides by enzymatic methods. The peptides are sequenced by the Edman-Dansyl Chloride technique and all fragments and peptides are then aligned in their proper order to yield the sequence of the whole chain.

2) Each of the major fragments has been characterized as to amino acid analysis and partial sequence. The fragments have been aligned in their probable order. Most of the tryptic peptides have been sequenced and many of them are aligned in their proper arrangement within the fragments.

3) One major amino acid change has been found so far between V-8 and Foggi: leucine 124 (V-8) is replaced by histidine (Foggi) without any detectable change in enzymatic properties. Moreover, a number of differences in amide group positions have been noted between the two strains.

Significance to Biomedical Research: The establishment of the sequence of this enzyme is fundamental in answering the question which is the major concern of this project, i.e., does the linear amino acid sequence, which is the primary expression of genetic information, contain in itself the ability to fold spontaneously to yield the native structure.

Proposed Course of Project:

1) Completely to establish the amino acid sequence of Foggi strain nuclease.

2) To find differences in the sequences of other strains of nuclease which may be discovered.

Serial No. NIAMD-LCB-11
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Proteolytic modification of staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-1

Principal Investigator: Dr. Hiroshi Taniuchi

Man Years

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

Objectives: To correlate covalent structure with the three dimensional structure and the enzymic mechanism of the nuclease.

Methods Employed and Major Findings: As reported in previous years the total amino acid sequence of nuclease-V8 (149 residues) has been elucidated and a new active derivative (nuclease-T) composed of two noncovalently bonded fragments has been discovered.

The step of the formation of nuclease-T by trypsin in the presence of thymidine diphosphate and calcium is as follows: trypsin cleaves the bond between residues 5 and 6 very rapidly forming two fragments, nuclease-T-P_1 (residues 1-5) and nuclease-T-P_{144} (residues 6-149). The latter possesses full enzymic activity and shows helical content and heat stability similar to those of intact nuclease. Subsequent tryptic cleavage of nuclease-T-P_{144} under the same conditions produces nuclease-T composed of nuclease-T-P_2 (residues 6-48) and nuclease-T-P_{3a} (residues 49-149) or nuclease-T-P_{3b} (50-149). These components obtained from both nuclease-V8 and nuclease-Foggi are exchangable to regenerate the enzymic activity. The restoration of helix upon association of nuclease-T-P_2 and -P_3 is also observed. Either of two components alone shows no significant helix by optical rotatory dispersion and circular dichroism.

Enzymically full active derivatives which are identical with or very similar to nuclease-T-P_{144} are obtained by limited digestions with α-chymotrypsin and subtilisin under the same conditions as described above.

The limited digestion of trifluoroacetylated nuclease with trypsin produces two fragments, TFA-nuclease-T-P_{126} (residues 1-126) and TFA-nuclease-T-P_{23} (residues 127-149). Neither of these fragments possess enzymic activity and mixing of the two shows no restoration of the activity. Study by circular dichroism measurement reveals essentially no helix in TFA-nuclease-T-P_{126}, indicating the importance of the carboxy-terminal residues for the three dimensional structure and the enzymic action of nuclease.

Significance to Biomedical Research: The object is to understand the significance of the amino acid sequence of protein controlled by the genetic information in terms of the three dimensional structure and function.

Proposed Course of Project:
1) Synthesis of covalent links between TFA-nuclease-T-P_{126} and -P_{23} in order to restore the enzymic activity.
2) Study of the three dimensional structure and function of protein lacking carboxy-terminal residues as an intermediate in biological protein synthesis.
3) Study interaction between nuclease-T-P_2 and -P_3.
4) Study relation between the three dimensional structure and function of intact nuclease and nuclease-T.

Publications:

Taniuchi, H., Anfinsen, C.B., and Sodja, A.: The amino acid sequence of an extracellular nuclease of Staphylococcus aureus. II. The amino acid sequences of tryptic and chymotryptic peptides. J. Biol. Chem. 242: 4736-4758, Oct. 1967.

Taniuchi, H., Anfinsen, C.B., and Sodja, A.: The amino acid sequence of an extracellular nuclease of Staphylococcus aureus, V8. III. Complete amino acid sequence. J. Biol. Chem. 242: 4752-4758, Oct. 1967.

Taniuchi, H., Anfinsen, C.B., and Sodja, A.: Nuclease-T: an active derivative of staphylococcal nuclease composed of two noncovalently bonded peptide fragments. Proc. Nat. Acad. Sci. 58: 1235-1242, Sept. 1967.

Serial No. NIAMD-LCB-12
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Large-scale preparation method of nuclease from Staphylococcus aureus (Foggi strain).

Principal Investigator: Dr. Ladislav Moravek

Man Years:

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

Objectives: Staphylococcal nuclease has become an important object for biological research. The absence of disulphide bridges, the relatively low molecular weight and its enzymological properties make it of particular value for understanding the relationship between its amino acid sequence, conformation and its enzymological properties. For these reasons nuclease has become subject to intensive study by a group of workers in the Laboratory of Chemical Biology. Its scarcity and high price however have become the limiting factor in further research. A decision was made to develop a new method for the isolation of larger quantities of nuclease at an acceptable price.

Methods Employed: Staphylococcus aureus was grown in 300 l fermenters at optimum conditions for the production of the extracellular nuclease. After the addition of Cetavlon the protein mixture was adsorbed on phosphorylated cellulose. In further purification steps proteins including nuclease were eluted from phosphorylated cellulose by 40% ammonium sulphate using a column arrangement. Subsequently the nuclease was precipitated by saturating the eluate with ammonium sulphate. The precipitate was desalted by dialysis. The resulting crude nuclease was then purified by gel filtration on a Sephadex G-75 column and by ion exchange chromatography on phosphorylated cellulose. The yield from one 300 l batch is approximately 2.5 g of pure nuclease.

Major Findings: A well-reproducible method has been developed, making possible isolation of relatively large quantities of nuclease. The application of this method on laboratory or commercial scale should considerably reduce the price of this protein. A detailed description of this method is being prepared for publication.

Significance to Biomedical Research: The availability of large amounts of this protein will facilitate the studies of its structure and biological function.

Proposed Course of Project: Possible improvements in the method are under investigation.

Serial No. NIAMD-LCB-13
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Tritium exchange in sperm whale and horse heart myoglobins and staphylococcal nuclease.

Principal Investigators: Dr. Ladislav Morávek
Dr. Alan N. Schechter

Man Years

Total: 1/2
Professional: 1/2

Project Description:

Objectives: Spectral, enzymatic, and chemical studies in this laboratory have given data on conformational differences between myoglobin and apomyoglobin (from sperm whale skeletal muscle and horse heart muscle) and changes in the conformation of nuclease upon the binding of cofactor and substrate (or inhibitor).

We wish to determine if the measurement of tritium exchange in these proteins will reflect these small changes in conformation and can be correlated with the previous data.

Methods Employed: Modifications of recently described methods (Englander in Poly α-amino Acids, ed. by Fasman. Dekker, 1967) have been developed for separating tritiated macromolecules from free THO and for following the kinetics of subsequent tritium exchange between macromolecule and medium. These methods include gel filtration in Sephadex and dialysis with continuous flow of the external buffer, from a specially constructed micro-dialysis apparatus, as well as appropriate methods for measuring protein concentration and radioactivity.

Major Findings: The equipment as now operating seems to be sufficient to allow meaningful measurement of all exchanging hydrogens in the above proteins. This arrangement also allows systematic variation in pH, ionic strength, ligand concentration, and other factors affecting protein conformation.

We have been able to label all of the exchangeable hydrogens of sperm whale metmyoglobin with tritium concentrations from 0.01 to 0.5 mc/ml and, by using interrupted gel filtration, have established the approximate sizes of the fast and medium exchanging pools.

Significance to Biomedical Research: It is now appreciated that enzymatic and other biological functions of proteins are determined by their three-dimensional conformations. Research in this laboratory is directed at elucidation of the factors which determine the conformation of proteins in solution. By correlating the results of tritium exchange with other physical and chemical methods, we hope to better define previously studied conformational change to obtain information about the utility and limitations of this method.

Proposed Course of Project: To the extent that tritium exchange is a sensitive method for measuring small changes in protein conformation, we will use this method for studying various proteins under conditions in which conformational changes have been hypothesized. The effect of the binding of heme to apomyoglobin and of the binding of nucleotides to nuclease will be examined first.

Serial No. NIAMD-LCB-14
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: X-ray studies on the three-dimensional structure of a nuclease from staphylococcal aureus.

Previous Serial Number: NIAMD-LCB-1

Principal Investigators: Christian B. Anfinsen
Hiroshi Taniuchi

Cooperating Unit: MIT (Drs. Cotton, Hazen, Richardson)

Man Years:
Total: 1/4
Professional: 1/4

Project Description:

Objectives: To investigate the molecular structure of nuclease by X-ray crystallographic methods.

Methods Employed and Major Findings: The cooperating unit at MIT has prepared several heavy atom derivatives of nuclease crystals that are isomorphous with the native crystals. The use of phases from one crystal form in the Fourier analysis of other forms has indicated the correctness of the phase selections. The various derivatives are sufficiently isomorphous that data is now being collected that will permit three-dimensional Fourier synthesis of the structure at a level of 4 Å resolution. Several derivatives of nuclease have been supplied to the MIT group lacking amino acid residues at the amino terminal end of the chain, and it is hoped that these partially degraded structures which crystalize in an isomorphous form will be of help to the MIT group in locating the spatial location of the amino terminal end of the molecule.

Significance to Biomedical Research: The availability of a three-dimensional structure for this protein will permit analysis of the relationships between structure and function in enzymic catalysis.

Proposed Course of Project: Collection of X-ray data and the preparation of further isomorphous derivatives will permit structural analysis to 2 Å resolution as judged by the excellence of the present data at lower resolutions. Variations in crystal structure will be studied as a function of the addition of certain competitive inhibitors to the native molecule.

Serial No. NIAMD-LCB-15
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Comparative studies of bacterial β-galactosidase isoenzymes.

Principal Investigator: Dr. Robert P. Erickson

Other Investigator: Dr. Edward Steers, Jr.

Man Years

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

Objectives: To study the various physico-chemical properties of several bacterial β-galactosidases in an attempt to elucidate the basis and nature of the various forms of this polymeric enzyme.

Methods Employed: Strains utilized were Aerobacter cloacae; Serratia marcescens, F-lac; Serratia marcescens, P-lac; Proteus mirabilis, F-lac; Proteus mirabilis, P-lac; Aeromonas formicans, and Streptococcus lactis. Strains were grown as flask cultures. The β-galactosidases in partially purified extracts have been studied for isoenzyme formation (by polyacrylamide disc gels), metal ion dependency, urea stability, and thermal inactivation. Aerobacter cloacae and P-lac bearing strains have been grown in preparative quantities (300 1 fermenter lots) and purified by previously described procedures (Craven, G.R., Steers, E. and Anfinsen, C.B.: J. Biol. Chem. 240: 2466-2477, June 1965).

Major Findings: The β-galactosidase of constitutive or fully induced E. coli K12 is seen on disc gels as a number of isoenzymes which represent the tetramer and higher aggregates. The β-galactosidase specified by the P-lac episome from P. mirabilis (studied in S. marcescens) forms isoenzymes which are more labile than those of E. coli and which have a higher ratio of tetramer to the other forms in fresh extracts. The difference in isoenzymes resides in the structure of the P-lac enzyme and is not due to a deficiency in some "aggregating factor" as S. marscescens carrying the F-lac episome from E. coli forms the E. coli isoenzymes. The β-galactosidase synthesized by an inducible strain of A. aerogenes does not form isoenzymes. The Aerobacter β-galactosidase has a sedimentation coefficient similar to that of the E. coli tetramer and will hydridize with it but differs in antigenic structure and amino acid composition. The β-galactosidases of Aerobacter and P-lac are less stable to thermal inactivation and urea denaturation than the E. coli enzyme with an order of stability: E. coli > P-lac > Aerobacter. The increased sensitivities correlate with a more rapid dissociation of the tetramer to the monomer. These data suggest that the intratetrameric bonds of the isoenzymes must have their structural basis in the intermonomeric bonds of the tetramer.

Significance to Biomedical Research: The results of these studies are leading to a clearer understanding of the forces involved in the general phenomenon of isoenzymes. The presumed role of multiple forms of enzymes in developmental processes has received considerable attention to date. The molecular basis for multiple forms is essential if we are to understand their significance in metabolic and developmental processes.

Proposed Course of Project: Complete purification of the Aerobacter and P-lac tetramers will allow characterization of the chemical basis for tetramer stability.

Serial No. NIAMD-LCB-16
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Multiple forms of the β-galactosidase of *E. coli* K_{12}.

Previous Serial Number: NIAMD-LCB-18

Principal Investigator: Dr. Sally L. Marchesi

Other Investigator: Dr. Edward Steers, Jr.

Man Years

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

Objectives: To study the various physico-chemical parameters of the β-galactosidase forms synthesized by *E. coli* K_{12} to characterize the structural basis of the various forms of this enzyme.

Methods Employed: The methods of isolation and purification of the isoenzymes are the same as has been described in previous reports (NIAMD-LCB-2, 1965) with the following modification: preparative amounts of highly purified isoenzymes are now obtained by gel filtration of the sephadex obtained material through sepharose 4-B columns (see Marchesi, S.L., Steers, E., Jr., and Shifrin, S., manuscript submitted to *J. Biol. Chem.*). The purified isoenzymes have been characterized employing polyacrylamide disc electrophoresis, analytical ultracentrifugation, amino acid analysis, electron microscopy, and urea and thermal inactivation.

Major Findings: The multiple forms of β-galactosidase exist as polymeric aggregates of increasing size ranging from 4N, 6N, 8N, 10N, 12N, and 16N units. The heavier aggregates (6N-16N) are more labile to a variety of treatments and dissociate through 4N and 2N particles becoming monomeric units (1N). The monomeric subunits of 1N are chemically identical in all species examined. Electron microscopy of the 4N, 6N, and 8N forms has revealed tetramers and "rosette" structures consisting of 6 and 8 units. These results demonstrate that the formation of higher aggregates does not involve a simple aggregation of the predominant 4N species as previously thought, but involve possibly dimers, and direct association of monomers. The biological basis for such forms is unknown at present, but remains a major experimental objective.

Significance to Biomedical Research: The results of these studies are leading to a clearer understanding of the forces involved in the general phenomenon of isoenzymes. The presumed role of multiple forms of enzymes in developmental processes has received considerable attention to date. The molecular basis for multiple forms is essential if we are to understand their significance in metabolic and developmental processes.

Proposed Course of Project: The study will require the isolation of each individual form of the isoenzymes for comparative purposes as well as a comparative analysis of the coli forms with those produced by other bacterial species (see Erickson & Steers, NIAMD-LCB-15).

Publications:

Steers, E., Jr., and Shifrin, S.: Characterization of the β-galactosidase from a lactose negative, complementing mutant of Escherichia coli K12. Biochim. Biophys. Acta 133: 454-462, April 1967.

Shifrin, S., and Steers, E., Jr.: The effect of urea of subunit interactions of β-galactosidase from Escherichia coli K12. Biochim. Biophys. Acta 133: 463-471, April 1967.

Serial No. NIAMD-LCB-17
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Isolation and characterization of undischarged and discharged trichocysts of Paramecium aurelia.

Principal Investigator: Dr. Janine Beisson

Other Investigator: Dr. Edward Steers, Jr.

Man Years

Total: 1 1/4
Professional: 1 1/4

Project Description:

Objectives: To successfully isolate undischarged trichocysts from the pellicle of Paramecium aurelia; to characterize the structural proteins involved in this structure and their possible functional role.

Methods Employed: Paramecia were grown in large batch culture (400 l lots) and harvested as described by: Steers, E., Jr.: Biochemistry 4: 1896-1901, Sept. 1965. The harvested cells were suspended in a mixture of sucrose, $Mg SO_4$ and dimethylsulfoxide which prevented the discharge of trichocysts. The cells were disrupted by homogenization in a Dounce homogenizer or by ultrasonic treatment. The cellular brei was processed by filtration through sephadex G-200 from which the undischarged trichocysts were collected in a purified form. Polyacrylamide electrophoresis and amino acid analyses were also performed on the solubilized proteins. Electron microscopic studies are underway on the intact trichocysts.

Major Findings: It has been possible for the first time to isolate in pure form trichocysts which remain intact and do not discharge. Electron microscopy of these structures has revealed two clearly different regions of structure together with a striation pattern only seen previously in the discharged trichocyst. Polyacrylamide electrophoresis on disc gels in the presence of 8 M urea reveals a highly complex pattern of approximately 15 bands. Amino acid analysis of these preparations has been completed with the usual complement of amino acids.

Significance to Biomedical Research: This work represents a continuation of our objectives to characterize structural proteins related to the cell surface and cell surface properties.

Proposed Course of Project: To fully characterize the protein components of the trichocyst, and study their role by comparison of several mutant forms which we have acquired.

Serial No. NIAMD-LCB-18
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Isolation and characterization of protein components of red cell membranes.

Previous Serial Number: NIAMD-LCB-19

Principal Investigator: Dr. Edward Steers, Jr.

Cooperating Units: Section of Macromolecular Biology (Dr. V.T. Marchesi and Dr. T. Tillack. NCI 5513)

Man Years

Total: 1/4
Professional: 1/4

Project Description:

Objectives: To study the physical, chemical and biologic properties of spectrin, a fibrous protein which we have isolated from the membranes of red cells. We hope to determine the molecular structure and chemical properties of this protein after isolation from human red blood cells in order to determine how it is organized in the intact membrane and what its functional role might be.

Methods Employed: Membrane fractions are prepared from red cells by an osmotic lysis procedure; they are extracted by several different procedures and as a result some of the membrane bound proteins are dissociated from the lipid complex of the membrane and appear in solution in aqueous buffers. Extraction media include ATP, β-mercaptoethanol, EDTA, and, in certain experiments, aqueous pyridine. The soluble proteins are concentrated by salt precipitation or pressure dialysis and further purified by column chromatography using Sephadex and Sepharose gels.

The purified proteins are further studied by electron microscopy using negative staining techniques and metal shadowing; polyacrylamide gel electrophoresis; free-boundary electrophoresis; analytical ultracentrifugation, and various immunoprecipitin reactions.

The proteins are further characterized by amino acid analysis, end group analysis, and peptide maps.

Major Findings: By subjecting isolated membranes of red blood cells to various extracting media made up of low ionic strength buffers, chelating agents, and β-mercaptoethanol we have solubilized approximately 25% of the total membrane-bound protein. This protein appears to be homogeneous by several physical criteria, and has the capacity to polymerize into helically coiled polymers which are identical in their ultrastructural appearance to actin, a fibrous protein of muscle. We established that this protein extracted from red cells was a specific component of the red cell membrane by showing that antibodies directed against this material did not react with serum protein or proteins released from red cells during hemolysis. We also found that antibodies against the membrane protein did not react with purified preparation of actin extracted from the same animals. We further showed that the ultracentrifuge and electrophoretic properties of the membrane protein also differed from those of muscle actin.

As a result of these studies we concluded that the protein extracted from the red cell membrane was a new species specific to the membrane and have named this component Spectrin (Science 159: 203, Jan. 1968).

Further studies of spectrin have shown that this protein does not have any bound lipid, carbohydrate, or nucleic acid, and appears to be a polymer unit in the native state. We have found that spectrin is present in all species of red blood cells so far examined (human, horse, rabbit, guinea pig, sheep and rat) and the proteins extracted from each species have the same electrophoretic properties. Also antibodies directed against guinea pig spectrin cross-react with spectrins extracted from cells of all the other species.

Preliminary amino acid analyses of spectrin extracted from several species also show that the total composition of these proteins is strikingly similar.

Significance to Biomedical Research: As a result of our studies using the red cell membrane we are now in a position to apply the same methods to studies of the protein components of membranes of nucleated cells.

Thus we now have some specific approaches which we can use to explore the surface properties of mammalian cells in our search to determine what chemical properties of cells are concerned with surface recognition and other cell-cell interactions. In addition to their relevance to future studies of malignant cell surfaces these findings may also suggest ways to study the chemical basis for certain diseases of red blood cells such as hereditary spherocytosis.

Proposed Course of Project: We hope to continue using the red cell membrane system as a basic experimental model in order to develop methods for extraction and purification of membrane components. In addition we now feel ready to apply some of the methods described above to other cell types and plan to start isolating and extracting cell membrane preparation of tissue culture cells.

We shall also continue with more detailed studies of the properties of spectrin and hope to determine its molecular weight, subunit structure, and certain aspects of its chemical composition.

We are also planning a detailed study of the sialic acid containing protein. This will involve developing methods for its purification. Since we feel that this protein or proteins may be involved in cell surface specificity as soon as we develop appropriate methods we will proceed directly to the study of its properties and distribution in cultured mammalian cells.

Publications:

Marchesi, V.T., and Steers, E., Jr.: Selective solubilization of a protein component of the red cell membrane. Science 159: 203-204, Jan. 1968.

Serial No. NIAMD-LCB-19
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Kinetic studies on repression of the histidine operon in *Salmonella typhimurium*.

Previous Serial Number: NIAMD-LCB-7

Principal Investigator: Dr. John S. Kovach

Other Investigator: Dr. Robert F. Goldberger

Man Years

Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives: A kinetic study of the process of repression of the enzymes for histidine biosynthesis was undertaken to gain some insight into the mechanism of the repression process.

Methods Employed: Various histidine auxotrophs of *Salmonella typhimurium* were grown under conditions in which histidine became a limiting growth factor in mid-log phase. After one hour, excess histidine was added to the culture, and the growth rate immediately returned to the fast rate characteristic of repressed cells. Aliquots were withdrawn from the culture medium periodically during the period of slow growth due to histidine limitation and following the addition of excess histidine. These aliquots were assayed for seven of the enzymes for histidine biosynthesis.

Major Findings: Following the addition of histidine to derepressed cultures of various histidine auxotrophs, the enzymes for histidine biosynthesis were found to become repressed in a definite sequence and with a definite pattern in which the temporal sequence corresponded with the positional sequence of the genes in the histidine operon. This correlation of time of repression and gene position was seen in all histidine auxotrophs, regardless of the mode of derepression. Thus, the kinetics of repression disclose phenomena which are different from those involved in derepression. For some time now, it has been tempting to present models for the mechanism of repression. However, the problem is complicated by the fact that we do not know at what level the repressor acts. Jacob and Monod have proposed that the repressor acts at the genetic level, whereas Ames and Hartman, on the basis of their studies of polarity in the histidine system, proposed that repression involves the translating process. Sequential repression of the enzymes for histidine biosynthesis lends support to the concept of repression control at the translation level. Quite aside from the possibility that the repressor may act at the genetic level (inhibiting the synthesis of the polycistronic messenger-RNA specific for the histidine operon), repression must also involve a cessation of attachment of ribosomes at the operator end of the message. The only way we can visualize a temporal sequence in the repression of the histidine enzymes is by stopping the translation of the histidine message first at the operator end and last at the distal end. According to this model, it must take approximately 15 minutes for the last ribosome which initiated translation to traverse the entire message.

More recently, we have found that the pattern of repression is altered when there is an alteration of the allosteric site of the first enzyme for histidine biosynthesis. When this site is rendered non-functional, either by the presence of a specific allosteric inhibitor or by mutation, then all of the enzymes for histidine biosynthesis became repressed simultaneously. The mechanism by which all parts of the polycistronic histidine messenger-RNA cease to be translated at the same time is not yet understood.

We conclude that the first enzyme for histidine biosynthesis is involved in regulation of translation of the histidine message. What is more, this involvement is realized through the allosteric site of the enzyme. Thus, a clear interrelationship has been established between the two most important control mechanisms for curtailing the biosynthesis of metabolites in bacterial cells -- repression and feedback inhibition.

Significance to Biomedical Research: Further understanding of the mechanisms involved in control of protein biosynthesis may have importance for the understanding of human physiology and disease.

Proposed Course of Project: The effects of various inhibitors of protein synthesis and of the histidine enzymes themselves will be tested, in an effort to more clearly define the mechanisms involved in the sequential and simultaneous repression of the histidine enzymes. Since it has become clear that the first enzyme for histidine biosynthesis is involved in regulation, the study will be extended to include isolation of this enzyme, and an *in vitro* study of its regulatory properties.

Publication:

Goldberger, R.F.: Patterns of repression and derepression of the histidine operon in *Salmonella typhimurium*. The Proceedings of the Symposium of Biologically Active Proteins and Nucleic Acids, Aug. 27-30, 1967, Hong Kong. In press.

Serial No. NIAMD-LCB-20
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Purification of the phosphatase-dehydrase enzyme from Salmonella typhimurium.

Previous Serial Number: NIAMD-LCB-5

Principal Investigators: Dr. Nanine S. Henderson
Dr. Sheldon Schlaff

Other Investigator: Dr. Robert F. Goldberger

Man Years

Total: 1
Professional: 1

Project Description:

Objectives: As part of a continuing study of the mechanisms of control in the histidine operon of Salmonella typhimurium, the isolation and characterization of several of the enzymes for histidine biosynthesis have been undertaken in order to obtain information not available from genetic studies. This project concerns the two enzymes, phosphatase and dehydrase, which catalyze the 9th and 7th steps of the pathway for histidine biosynthesis, respectively. Although the enzymes are distinct in terms of their catalytic activities, their structures are determined by a single gene in the histidine operon. It is likely that they are part of one physically integrated unit, composed of several polypeptide chains. One of the objectives of this project is to study the structure of this double enzyme in order to elucidate the physical basis for the genetically defined complementation groups within the corresponding gene.

Methods Employed: Large scale growth of bacteria under conditions in which the histidine operon is derepressed approximately 30-fold. Preparation of cell-free extracts. Fractionation with acetone and with ammonium sulfate. Ion-exchange chromatography on DEAE-Sephadex. Continuous-flow high voltage electrophoresis. Sucrose density gradient centrifugation on a preparative scale. Electrophoresis in polyacrylamide gels. Assays for enzymic activities.

Major Findings: Purification of the enzyme(s) was extremely difficult, with erratic behavior on chromatography which often resulted in bizarre elution patterns. It finally became clear that the enzyme(s) exists in several forms which are in rapid equilibrium with one another under a wide variety of conditions. Thus, the molecular weight of the partially purified protein could be estimated at 150,000 and 75,000, and, under certain conditions, at 45,000. The impression from genetic studies that the _B_ gene of the histidine operon specifies the structure of a single protein with two enzymic activities appears to have been born out by the present study. At no time was it possible to dissociate the two activities, despite the fact that it was possible to reversibly denature the enzyme(s).

Significance to Biomedical Research: As part of a larger study on control mechanisms in protein biosynthesis, the project may have important implications for an understanding of cellular physiology in general.

Proposed Course of Project: Terminated.

Serial No. NIAMD-LCB-21
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Kinetics of induction of enzymes of an F'lac in Salmonella typhimurium.

Principal Investigator: Dr. Antonio Ballesteros

Other Investigators: Dr. John S. Kovach
Dr. Robert F. Goldberger

Man Years

Total: 1 1/2
Professional: 1 1/2

Project Description:

Objectives: This study was undertaken in order to examine the question of whether our previous findings on the kinetics of derepression of the enzymes for histidine biosynthesis were of general applicability. In order to maintain the same genetic background used in the previous studies, episome from E. coli, carrying the lactose operon, was introduced into Salmonella typhimurium.

Methods Employed: Salmonella typhimurium F'lac was grown in the presence of excess histidine and with glycerol as carbon source. The lactose operon was induced by the addition of IPTG to the culture medium. Samples were removed from the culture periodically during the periods immediately preceding and following induction, and these samples were assayed for β-galactosidase and transacetylase -- the enzymes specified by the first and third genes, respectively, of the lactose operon. The same experiment was repeated with both the wild type organism and the auxotroph hisG52, in the presence and absence of compounds known to decrease or increase the formylating capacity of the cell.

Major Findings: We have found that the wild type organism (which displays the sequential mode of derepression of the histidine enzymes) and hisG52 (which displays the simultaneous mode) both display a pattern of induction of the lactose enzymes which is essentially simultaneous. However, as in the case of the histidine system, reducing the formylating capacity of the cell results in sequential induction of the lactose enzymes.

Significance to Biomedical Research: As part of a larger study on control mechanisms in protein biosynthesis, the project may have important implications for an understanding of cellular physiology in general.

Proposed Course of Project: The study will be continued to determine whether the two modes of induction already observed are truly dependent upon the formylating capacity of the cell.

Serial No. NIAMD-LCB-22
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Alternative modes of derepression of the histidine operon in *Salmonella typhimurium*.

Previous Serial Number: NIAMD-LCB-6

Principal Investigators:
Dr. Mary Anne Berberich
Dr. Robert F. Goldberger
Dr. John S. Kovach
Dr. Warren Davis

Man Years

Total: 2 1/4
Professional: 1 3/4
Other: 1/2

Project Description:

Objectives: In order to gain some insight into the mechanism of control of a polycistronic operon *in vivo*, we have investigated the initial kinetics of derepression of the histidine system. This study was done for the purpose of testing our previous hypothesis that the two modes of derepression displayed by the various histidine auxotrophs reflect different mechanisms by which translation of the messenger-RNA for the histidine operon is initiated.

Methods Employed: Various histidine auxotrophs of *Salmonella typhimurium* were grown under conditions in which histidine became a limiting growth factor in mid-log phase. The histidine operon is known to derepress when this occurs. Aliquots were withdrawn from the cultures periodically before and after derepression, and extracts prepared from these aliquots were assayed for protein and for 7 of the enzymes for histidine biosynthesis. Experiments of this type were performed on a large number of histidine auxotrophs, under conditions in which the formylating capacity of the cell had been decreased (by the addition of trimethoprim or sulfacetamide to the culture medium) or increased (by the addition of compounds known to enrich the one-carbon pool of the cell -- compounds such as adenine, serine, and methionine). The kinetics of derepression were also studied in a double mutant, blocked in both the histidine pathway and the purine pathway.

Major Findings:
1) Histidine auxotrophs with a block in the histidine pathway which

results in the accumulation of phosphoribosylaminoimidazole carboxamide (PR-AIC) have a greatly diminished formylating capacity, and display the sequential mode of derepression; those with a block which does not result in the accumulation of PR-AIC have a normal capacity to carry out formylation reactions, and display the simultaneous mode of derepression.

2) When the PR-AIC accumulated in certain mutants is not allowed to lower the formylating capacity of the cell, then the mode of derepression is simultaneous, just as though no PR-AIC had been accumulated. This finding was obtained by the use of a double mutant, containing, in addition to the *his* mutation, a mutation preventing the conversion of PR-AIC to formylPR-AIC.

3) Compounds which diminish the formylating capacity of the cell by interfering with the synthesis of the carrier for formyl groups cause the mode of derepression to be sequential regardless of which mutant is examined.

4) Compounds which increase the formylating capacity of the cell by enriching the one-carbon pool (that is, compounds which participate in one-carbon transfer reactions or which themselves require one-carbon addition for their own biosynthesis) cause the mode of derepression to be simultaneous regardless of which mutant is examined.

The hypothesis that the mode of derepression depends upon whether translation of the histidine messenger-RNA is initiated at the operator end only or at multiple sites can now be linked with the finding that the mode of derepression depends upon the formylating capacity of the cell. The element which links the two together is the finding, in several laboratories, that N-formylmethionyl-tRNA is required for the initiation of the polypeptide chains of all bacterial proteins. Evidently, when the supply of formyl groups is limited, the availability of N-formylmethionyl-tRNA for polypeptide chain initiation is restricted, and translation of the histidine message is initiated at the operator end preferentially. When the supply of formyl groups is not limited, a sufficient amount of N-formylmethionyl-tRNA is available so that translation of the histidine messenger-RNA may be initiated at multiple sites. These findings provide evidence for the first time that punctuation exists, *in vivo*, in a polycistronic message, allowing certain codons to signal the initiation of translation despite their internal positions.

Significance to Biomedical Research: Further understanding of biological control may have importance for the understanding of human physiology and disease.

Proposed Course of Project: The study will be extended to a study of polar mutants of the histidine operon, in an effort to gain further insight into the mechanism by which translation of a polycistronic messenger-RNA is initiated and into the phenomenon of polarity itself.

Publications:

Berberich, M.A., Kovach, J.S., and Goldberger, R.F.: Chain initiation in a polycistronic message: sequential versus simultaneous derepression of the enzymes for histidine biosynthesis in *Salmonella typhimurium*.

Proc. Nat. Acad. Sci. U.S. 57: 1857-1864, June 1967.

Margolies, M.N., and Goldberger, R.F.: Correlation between mutation type and the production of cross-reacting material in mutants of the *A* gene of the histidine operon in *Salmonella typhimurium*. *J. Bact.* 95: 507-519, Feb. 1967.

Serial No. NIAMD-LCB-23
1. Lab. of Chemical Biology
2. Genetics and Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Human red blood cell phosphoglucomutase.

Previous Serial Number: NIAMD-LCB-15

Principal Investigator: Dr. Robert E. Coifman

Man Years

Total: 3/4
Professional: 3/4

Project Description:

Objectives: Many human tissues, of which red blood cells are the most readily available for study, contain four or more electrophoretically distinguishable forms of the enzyme phosphoglucomutase. These enzymes are the products of two separate genetic loci, which appear to segregate independently. The goal of this project is to isolate and purify sufficient quantities of the several enzyme forms to permit comparative chemical and structural studies.

Methods Employed and Major Findings: Phosphoglucomutase is extracted from surplus or outdated blood bank red cells after preliminary genotype determination by starch gel electrophoresis. The availability of quantities on the order of 50 units for a batch preparation is a product of the needs of the NIH Clinical Center for fresh, platelet-rich plasma and makes a study of this type technically feasible at NIH while it would not be at several centers currently investigating phosphoglucomutase from non-human sources.

Many of the obstacles have now been overcome in the adaptation of a separatory method employing adsorbtion to substituted cellulose columns and the relative heat resistance of this enzyme to isolation of the enzyme on a preparative scale. As this report is being written (May 4) a lot is nearing the end of a preparative sequence which should permit a number of chemical comparisons to be made of the five principal electrophoretic bands found in the most common homozygous electrophoretic genotype.

Significance to Biomedical Research: A considerable literature, some of it conflicting, has been published concerning the classical and molecular biochemistry of phosphoglucomutase. Though almost all of this work has been performed with enzyme from non-human sources it appears that certain homologies exist in species as diverse as man, bacteria, and higher plants, all of which produce two different single polypeptide chain phosphoglucomutases of roughly similar molecular weight, each of which exists in both phospho- and dephospho- forms.

While the use of starch gel electrophoresis for phosphoglucomutase genotype determination is common in the field of human population genetics and an incompletely investigated degree of electrophoretic heterogeneity also exists in other species, electrophoretic identification of the usually differently prepared rabbit muscle enzyme preparations on which most of the biochemical literature is based has with one exception never been reported. The most immediate significance of this project to biomedical research is the opportunity it contains to establish a correspondence between existing knowledge of the biochemical genetics of this enzyme on the one hand and of its population genetics on the other.

Proposed Course of Project: Several attempts to interconvert what at simplest interpretation should be corresponding phospho- and dephospho- electrophoretic fractions in experiments conducted without the use of radioisotopes have been uniformly unsuccessful. The next scheduled phase of the project is the adaptation and quantitation of a technique for the preparation of ^{32}phosphorus-labeled substrate. Depending on the availability of time a series of tracer enzyme-labeling experiments may either be started during the period covered by this report or else performed at a later date. The feasability of other chemical studies employing the lot of enzyme currently in preparation will depend on its ultimate chemical purity.

Serial No. NIAMD-LCB-24
1. Lab. of Chemical Biology
2. Genetics and Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The population genetics of thymic alymphoplasia.

Principal Investigator: Dr. Robert E. Coifman

Man Years

Total: 1/4
Professional: 1/4

Project Description:

Objectives: To develop and test a method of numerical analysis capable of estimating separate autosomal recessive and x-linked recessive gene frequencies, number of independent autosomal recessive genes capable of producing a condition, and the probability with which any given affected family or individual has autosomal recessive or x-linked disease from a series of case reports without population incidence data.

Methods Employed and Major Findings: A series of 44 families with either single or multiple incidence of thymic alymphoplasia has been drawn from the personal experience of the principal investigator and the published literature. A computer program was developed for the iterative estimation of autosomal recessive and x-linked gene frequencies, based on the increased amount of inbreeding in the families of patients assignable as autosomal recessive with respect to estimates for the population at large and the sex ratio of patients in the simplex families. The program is currently being modified to permit the simultaneous estimation of the number of independent autosomal recessive genes active in causing this disease in the population.

Significance to Biomedical Research: This technique is potentially applicable to the study of a number of diseases in which an indistinguishable clinico-pathologic final common path results from presumable different genetic abnormalities with autosomal recessive and x-linked recessive hereditary patterns. It may prove to be less applicable than "classical" population genetics methodology for traits in which there is a significant non-genetic or sporadic occurrence (which does not appear to be the case for thymic alymphoplasia) but it does not require the population incidence data necessary for the latter if a series of probands can be assumed to be representative of the distribution of the disease in the population.

This project can contribute to the understanding of investigations into the pathobiology of this disease by providing an estimate of the number of separate genetic abnormalities with which an investigator may be confronted in a study of material from a series of patients, and, when pooling or grouping of specimens from several sources may be necessary to obtain sufficient material for certain studies it may be of value in suggesting which groups of patients may be how likely to have what number of separate genetic disorders.

Proposed Course of Project: This project will be continued by the present author during the coming fiscal year at the University of Minnesota, where it will be expanded to include the larger series of cases on file at that institution. To accommodate this larger series the existing program will have to be translated from BASIC, in which form the present series occupies the full capacity of the General Electric-Datanet time sharing, on line, remote access computer, to FORTRAN for use on larger machines. If a set of data suitable for analysis by both methods can be found this technique will also be compared directly with so-called "classical" techniques.

Serial No. NIAMD-LCB-25
1. Lab. of Chemical Biology
2. Genetics and Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Protein differentiation during embryogenesis

Previous Serial Number: NIAMD-LCB-16

Principal Investigator: Dr. P.V. Tishler

Man Years

Total: 1
Professional: 1

Project Description:

Objectives: The present study is aimed at a correlation of changes in types of proteins synthesized, as well as changes in the kinetics of metabolism of such proteins, with morphogenesis of organs or their anlage in the fetal mouse. It is hoped that such an approach will yield information concerning the interrelationships of genes, and their products, which are important in normal mammalian differentiation. In addition, certain genetically delineated mutations which prevent normal differentiation in the mouse will be studied in a similar manner, with the aim of ascertaining whether these mutations result from the synthesis of abnormal proteins.

Methods Employed and Major Findings:

1) Obtaining experimental animals - the Balb/c inbred strain of mouse has been selected for study, since the NIH strain is both hardy and prolific. Three mutations, obtained from workers outside NIH, are being introduced separately into this strain by techniques which will ultimately create coisogenic sublines. These mutations are brachyury, an autosomal dominant which prevents normal posterior notochordal-neural tube differentiation; syndactylism, which causes fusion of toes by localized ectodermal overgrowth in the early limb bud; and brachypodism, which limits the length of all extremities by retarding the rate of chrondrogenesis in the limb bud.

Because large numbers of embryos of specific gestational ages will be needed in subsequent experiments, studies were initiated with the Balb/c strain of mouse to determine conditions permitting the synchronization of estrous cycles and successful matings within a narrow time interval. Other workers had demonstrated that appropriate perenteral dosages of pregnant mares' serum (primarily FSH) and of human corionic gonadotropin accomplished these aims in many strains of mice. Such hormonal priming has been unsuccessful thus far with the Balb/c, however, despite a systematic study of many of the variables of this method. Presently, it would seem that the female Balb/c is exquisitely sensitive to olfactory influences (pheromones) from males other than the stud, and that these prevent implantation of the embryos in the uterus.

2) Methodology of separation and comparison of tissue proteins - much of the past year has been spent in developing the following methodology, and delineating its efficacy and limitations. Basically, the synthesis and turnover of various proteins from selected organs or their anlage will be assessed by incorporation of radioisotope. It has been found that sufficient amounts of isotope (either cabron-14 or tritium) may be incorporated into fetal protein by intraperitonal injection of the gravid female with radioactive amino acids. Proteins are extracted in either aqueous medium (water seems to be a superior extractant to either sucrose or buffer, regardless of dilution of the latter) or 8 M urea. About seventy per cent of total tissue protein may be thus extracted. Initial separation of proteins is effected by thin layer gel filtration on Sephadex G-200 superfine. When 100 ug of protein from any of the tissue extracts is allowed to migrate 20 cm on a 0.5 mm layer of gel, the proteins are effectively separated into a continuum of species. Fractions of protein with similar migrations on the Sephadex may be removed with a spatula, and each gel "cut" is then layered on top of a standard polyacrylamide gel. After discontinuous electrophoresis, each gel may be stained for protein, or it may be sliced and each slice assessed for radioactivity by methods developed in this laboratory.

It is presumed that the method outlined above will serve as an adequate screening procedure with which to evaluate protein synthesis in such tissues as neural tube and limb bud, in both normal and mutant embryos, and during various chronological stages of embryogenesis. Should differences in protein metabolism be found by this correlative method, other techniques may be brought to bear to determine their nature and significance.

The methodology outlined above has been tested by comparing the protein patterns (by stain) obtained from livers of adult mice of several inbred, virtually unrelated species. Of some sixty protein bands in each of the specimens, each differs from the others by the presence, absence or quantitative variation of only one or two proteins. The nature of these differences is presently under investigation.

Significance to Biomedical Research: In addition to providing some insight into the role of proteins and protein synthesis in normal differentiation, this project may shed light on etiologic factors involved in abnormal differentiation associated with hereditary diseases of both mice and men.

Proposed Course of Project: To be continued indefinitely.

Serial No. NIAMD-LCB-26
1. Lab. of Chemical Biology
2. Genetics and Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Factors determining the conformation of proteins in solution.

Previous Serial Number: NIAMD-LCB-11

Principal Investigator: Dr. Alan N. Schechter

Cooperating Unit: LTD,NHI (Dr. R. Chen)

Man Years
Total: 3/4
Professional: 3/4

Project Description:

Objectives: The role of ligands in modifying the amino acid sequence-determined conformation of proteins has become more fully appreciated in the last few years. In proteins with subunits, the interaction of the separate polypeptide chains also refines the structural and functional properties of the molecules.

We have continued and extended our investigations into the factors determining conformation of proteins in solution by studying the physical and chemical properties of myoglobin and malic dehydrogenase.

Methods Employed: Chicken heart malic dehydrogenase has been isolated, purified, and partially resolved into its seven or more isoenzymes by ammonium sulfate fractionation, CM-cellulose, and DEAE-cellulose chromatography. Conditions have been established for the regeneration, from 7.6 M guanidine hydrochloride, of as much as 95% of initial enzyme activity. Using this reversible denaturation method and enzyme activity stains on starch gel electrophoresis, the question of conformational isoenzymes has been investigated.

Chromatographically purified sperm whale and horse heart myoglobin has remained as our second major object of investigation. This has been studied in the apo- form (with heme removed by acidified acetone), as well as in the met-myoglobin state. We have made further use of the absorbance, fluorescence, and optical rotation methods. Also, we have begun investigation of the physical and chemical properties of apomyoglobin reconstituted with other ligands, such as anilino-naphthalene sulfonate and protoporphyrin IX. Although most of these measurements, with heme and other ligands, were done at equilibrium, kinetic work has been performed with a newly devised stopped-flow instrument.

Major Findings: Our study of the reversible denaturation of the chicken heart malic dehydrogenase isoenzymes has led to the conclusion that interconversion of the electrophoretically different bands does not occur under conditions of random coil behavior, when conformational heterogeneity would be eliminated. However, there is a decreasing efficiency of enzyme recovery from cathodal to anodal components. These results, in conjunction with other experiments, indicate that there are covalent differences among these isoenzymes and that the conformer hypothesis is not an adequate explanation of this system.

The studies of the urea- and guanidine hydrochloride-induced denaturation of metmyoglobin and apo-myoglobin have been collated and analyzed in terms of the molecular model and the thermodynamics of denaturation. The data demonstrate a one-step denaturation process under all conditions studied, and are consistent with Tanford's model of the denaturation process. Indeed, this analysis leads to a value of 15 Kcal/mole of stabilization free energy for the heme binding, in accordance with other measurements.

Kinetic measurements of the reconstitution process continue to point to the existence of a special stable intermediate:heme bound to the denatured protein. Measurements of the quantum yield of tryptophan fluorescence of metmyoglobin in concentrated denaturant and the ultracentrifugation properties in denaturant, however, indicate that such a fraction must compose less than 5% of the total.

A more detailed study of the kinetic aspects of the denaturation and renaturation processes has been made possible by our developing a stopped-flow instrument that may be used in conjunction with standard spectrophotometers and spectrophotofluorometers. This instrument, whose description has been prepared for publication, consists of a yoke and driving block powered by compressed air and a quartz micro-cuvette with appropriate mixing and stopping devices. The dead time of the instrument can be made as low as 20 msec., but for ordinary work it is about 50 msec. With it we have studied reactions, including enzyme catalysis as well as denaturation, with half-times of the order of seconds.

A method for measuring interaction among chromophores has been used, in conjunction with Dr. R. Chen, to determine aspects of the structure of myoglobin in solution. Ligands, including anilino-naphthalene-sulfonate, dansyl sulfonamide, and protoporphyrin IX have been bound to apomyoglobin. The stoichiometry and association constants of these reactions, as well as other evidence, indicates that these ligands bind in the heme "pocket." Using fluorescence and absorbance spectroscopy we have been able to calculate the efficiency of energy transfer between these ligands and the tryptophans of the protein. This information has been used with the Förster theory to solve for the distance separating the chromophores. The results are in accordance with that predicted from the atomic model.

Significance to Biomedical Research: The results of these studies on protein conformation further our understanding of the factors contributing to determining the conformation of proteins in solution, especially the role of the interaction with ligands. There is increasing realization that small conformational changes may have significant functional effect in protein, including enzyme, systems. Our work may lead to better appreciation of such fine-structural changes.

Proposed Course of Project: As soon as these studies can be prepared for publication, with any necessary corollary work, they will be concluded. Studies of the interaction among amino acid sequence and ligands in determining conformations will be continued in the measurements of tritium exchange (with Dr. Moravek) and peptide interactions (with Dr. Taniuchi).

Publications:

Schechter, A.N., and Epstein, C.J.: Mitochondrial malic dehydrogenase: reversible denaturation studies. Science 159: 997-999, March 1968.

Schechter, A.N., and Epstein, C.J.: Spectral studies on the denaturation of myoglobin. J. Mol. Biol., in press.

Serial No. NIAMD-LCB-27
1. Lab. of Chemical Biology
2. Genetics and Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The effect of Ca on the renaturation of reduced taka amylase.

Previous Serial Number: NIAMD-LCB-14

Principal Investigator: Dr. Theodore Friedmann

Man Years

Total: 1/4
Professional: 1/4

Project Description:

Objectives: This study continues the studies on the forces directing and stabilizing the tertiary structure of globular proteins. It has been found that the neutral ligand Ca^{++} is required for the full reoxidation of reduced Taka amylase. This protein normally contains 4 disulfide bonds, and full reoxidation under optimal conditions in the presence of Ca^{++} results in completely native-like enzyme with full enzymic activity and normally paired half cystine residues. In the absence of Ca^{++}, only 3 of the 4 disulfide bonds in the enzymically inactive form are formed, but these have normal half cystine pairing. The subsequent addition of Ca^{++} leads to the oxidation of the last disulfide bond and the appearance of enzymic activity. The effects of Ca^{++} on the tertiary structure of the partially reoxidized amylase have been studied now by physical methods.

Methods Employed: The effect of Ca^{++} on the conformation of partially reoxidized amylase has been investigated by solvent perturbation spectroscopy, ordinary UV difference spectroscopy, and fluorescence spectroscopy. The disulfide bond which participates in the conformational transitions has been partially characterized by preparative diagonal electrophoresis, amino acid analysis and end group analysis.

Major Findings: Ca^{++} has been shown to cause a conformational change of partially reoxidized amylase and such a change is accompanied by the "burial" of approximately 9 tyrosine residues, indicating that the change in conformation involves significantly large portions of the polypeptide chain. The disulfide bond which is stabilized by Ca^{++} is partially characterized, and shows an unusual amino acid composition, containing large numbers of serine and threonine residues.

Significance to Biomedical Research: This study provides some further understanding of the nature of protein folding.

Proposed Course of Project: The full amino acid sequence of the disulfide peptide will be determined, and an attempt will be made to identify the nature of the Ca^{++} binding site.

Publications:

Freidmann, T. and Johnson, P.: The structure of Tamm-Horsfall mucoproteins in fibrocystic disease. J. of Lab. and Clin. Med. 70: 404-407, Sept. 1967.

Friedmann, R., and Epstein, C.J.: The role of calcium in the reactivation of reduced Taka-amylase. J. Biol. Chem. 242: 5131-5140, Nov. 1967.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMICAL PHARMACOLOGY

The Biochemistry of Polysaccharides and Glycoproteins

(a) Changes after TSH Injection and in Exophthalmus

Injection of TSH to guinea pigs produces (1) an increase in $^{35}SO_4$ uptake by Harderian glands (increased sulfate is mainly in the dialysable fractions after a single TSH injection, but there is also increased sulfate in the mucopolysaccharide fraction if TSH is injected daily for 3 and 5 days); (2) an accumulation of mucopolysaccharides by Harderian gland which is maximal after 3 and 5 days of daily injections but disappear after 15 days; (3) a modification in wet weight which parallels the level of mucopolysaccharide and the appearance and disappearance of visible exophthalmos; and (4) an increase in $^{35}SO_4$ uptake and mucopolysaccharides content in the perirenal fatty tissue and intestine but not in the skin and muscle. All the mucopolysaccharides normally present in Harderian gland are proportionally increased after TSH injection.

The excretion of mucopolysaccharides normally found in urine (chondroitin, chondroitin sulfate A and C, keratosulfate, and heparitin sulfate) is increased approximately twofold in patients with progressive exophthalmos. A threefold elevation of total serum mucopolysaccharides is also found. These increases are unrelated to thyroid function.

(Dr. Roger J. Winand)

A separation of TSH and exophalmic factor activities has been explored. Preparations have been resolved into single protein species by disc gel analyses, with individual components retaining both activities to differing degrees.

(Drs. Roger J. Winand and Leonard D. Kohn)

(b) Studies on Blood Group Substances.

Differences in the structure of carbohydrates which occur on the surface of red blood cells and in secretions such as saliva and milk are responsible for differences in certain blood types that distinguish humans. It has been found that the presence or absence of specific transferases in individuals is genetically controlled and determines the structure of these complex carbohydrates and hence the blood type of the individual. Therefore, unlike proteins whose structure is directly determined by genes and are primary gene products, the structural determinants of blood type can be considered to be secondary gene products. That is to say, the direct products of the genes that determine blood type are enzymes and it is these enzymes working in concert that are responsible for the formation of the specific antigenic structures This type of mechanism explains the well-known fact that individuals can have cell surface antigens not possessed by either parent and provides a possible mechanism for the expression of individuality. The four transferases that have been demonstrated thus far are responsible for the presence or absence of six blood group specificities: type XIV, Le^a, Le^b, O(H), A, and B.

(Drs. Evelyn F. Grollman, Akira Kobata, Laura Shen, and Victor Ginsburg)

Ribosomal Structure and Function

This laboratory has been investigating the mechanism of polypeptide synthesis *in vitro* using ribosomes and enzymes from *Escherichia coli*. Essentially two approaches have been used, as follows.

(1) The biological and chemical nature of functional ribosomes has been examined by studying the behavior *in vitro* of ribosomes that have been modified either chemically (by selective chemical reagents) or genetically. This work has been facilitated by the development in this laboratory of methods to prepare very highly purified and stable ribosomes.

(2) As an integral part of the first problem studies have been made on the interaction of ribosomes with the enzymes (transfer enzymes) that in some way catalyze polypeptide synthesis. In order to carry out this work we had to purify the transfer enzymes; in the process of doing so, we found that the previously described transfer enzymes (Ts, Tu, and G) may be derived from a single protein entity. We were able to stabilize this protein entity, which we call E-1, and were able to purify it extensively. During purification we were able to separate from E-1 a low molecular weight substance which we have called E-2. E-1 and E-2 are required for polypeptide synthesis with our highly purified ribosomes.

(Drs. Anthony V. Furano and Vincent W. Hollis, Jr.)

Yeast Thiol Disulfide Transhydrogenase

This enzyme catalyzes the reduction of L-cystine by glutathione, and it is postulated to play an *in vivo* role in preventing the intracellular accumulation of cystine such as occurs in the disease cystinosis. Work is proceeding to perfect a purification procedure which was developed by Dr. Nagai and to devise methods for a study of its catalytic mechanism. The mechanism is considered to be complex, involving several intermediate steps.

(Drs. Simon Black, Sadamu Nagai, and Laurence D. Aronson)

Yeast Glycyl tRNA Synthetase

This enzyme activates the amino acid glycine and catalyzes the attachment of its molecule to one of tRNA (transfer ribonucleic acid). Factors involved in maintaining this enzyme's activity at a maximal rate are being investigated as possible physiological regulators. It has been possible to prepare the enzyme in a condition such that it maintains a constant rate of glycyl tRNA synthesis for a period of two hours. This is to be contrasted with the usual preparations of amino-acyl tRNA synthetases which maintain original rates for only 10 to 20 minutes.

(Dr. Simon Black, Mrs. Blondel Hudson, and Mrs. Gloria Johnson)

Phage λ DNA Synthesis

Drs. David Korn and Doris Dahl have continued the investigation begun a year earlier with Dr. N. L. Couse into the mechanism of λ DNA replication. Under the conditions of λ infection of *Escherichia coli* pulse-labeled phage DNA is initially isolated as a very rapidly sedimenting complex with $S > 1{,}000$. Techniques have been developed for releasing the DNA from the complex. Results thus far indicate that the isolated pulse-labeled λ DNA sediments together with purified λ marker DNA in both neutral and alkaline sucrose gradients. Thus they find no evidence of closed circular DNA molecules or of DNA "concatenates." The data also indicate that some of the "unusual" DNA forms that have been described by other workers may well be the result of artifacts introduced by the methods used both to lyse infected cells and to extract pulse-labeled DNA. The two major aims of this study are (1) to describe the structure of nascent λ DNA and (2) to investigate the properties of the rapidly sedimenting complex with which the nascent DNA appears to be very avidly associated.

(Drs. David Korn and Doris Dahl)

DNA Polymerase from KB Cells

For the past year Drs. David Korn and Richard Greene have been attempting to purify and characterize the DNA polymerase from a mammalian cell system, KB cells, as an initial approach to the problem of adenovirus replication in these cells. The problem has been complicated by the extreme lability of the partially purified enzyme, but they have now devised a procedure that yields polymerase in good yield at about a thousand-fold apparent purification. The purified preparation contains only a single major protein band by analytical disc gel electrophoresis. They have partially characterized this enzyme.

(Drs. David Korn and Richard Greene)

The Inhibition of Bacteriophage T_4

In continuation of Dr. Korn's studies of the mechanism of selective inhibition of bacteriophage T_4 maturation by actinomycin D, he has isolated a number of T_4 mutants that are partially resistant to this drug. Dr. Korn has mapped four of these mutants in a single cistron that is known from the work of Edgar *et al.* to be concerned with the assembly of phage components and the packaging of phage "replicating" DNA within phage heads. Further work with these mutants will be directed toward attempting to define the mechanism of DNA packaging and the function of this "assembly locus."

(Dr. David Korn)

Studies on the Surface Components of *Escherichia coli*

Alterations produced by EDTA in the surface of *Escherichia coli* include a general increase in permeability and release of surface material, including about half of the cell's lipopolysaccharide and smaller amounts of phospholipid and protein. These will be studied as follows.

(1) Chemical Changes in the Cell Surface

Previous work has shown that the lipopolysaccharide released by EDTA treatment contains two fractions which can be separated by ultracentrifugation. One fraction is associated with released phospholipid and protein, and one is not. The two fractions also differ in composition. Studies have been carried out defining the relationship of these fractions to the rest of the cell surface by (a) pulse labeling to determine the metabolic relationship of these fractions with the lipopolysaccharide retained by the cell and (b) by further chemical characterization of the protein and phospholipid associated with this lipopolysaccharide.

(Drs. Loretta L. Leive and Stuart B. Levy)

(2) Relationship of Chemical Changes to the Permeability Change

It is difficult to determine whether the chemical changes described above are the cause of, effect of, or unrelated to, the permeability change. We have therefore isolated mutants (a) that show increased permeability without EDTA treatment and (b) that are resistant to the EDTA-caused permeability change. Further study of these is being carried out to determine which chemical changes are responsible for the permeability increase.

(Drs. Loretta L. Leive and Mary Jane Voll)

Bacteriophage-Induced Nucleases

Two enzymes which degrade denatured DNA, a polymerase associated nuclease and an oligonucleotide diesterase, have been shown previously in this and other laboratories to be produced after infection of *Escherichia coli* with T_{even} bacteriophage. We have now shown that the two enzymes can be distinguished on the basis of the relative rates of hydrolysis of DNA molecules of different sizes, since only the second enzyme degrades very short oligonucleotides ($n = 4$) at an appreciable rate.

A chromatographic procedure has been developed to stabilize and rapidly separate these two nucleases. Using this procedure we have examined many amber mutants of T_4 for changes in DNA polymerase and DNase activities. Most mutants of gene 43, shown by others to code for the polymerase, lack both the polymerase and the associated nuclease. The oligonucleotide diesterase is present at normal levels in these mutants, suggesting that the two nucleases are the products of different genes. One mutant in gene 43 has been found which lacks the polymerase but contains what appears to be a modified polymerase-associated nuclease. Mutants in genes 46 and 47, which cannot degrade bacterial DNA, have normal levels of both the nucleases studied here, implying that still other enzymes are required for this function.

(Dr. Nancy G. Nossal)

Structure-Function Studies on "Hydroxypyruvate Utilizing" Reductases

Structure-function comparisons of "hydroxypyruvate utilizing" reductases have been initiated. Glyoxylate reductase, tartronic semi-aldehyde reductase, and hydroxypyruvate reductase have been found to have a subunit structure. The former enzyme M. Wt. 100,000 is composed of three subunits, M. Wt. 36,000. Subunit arrangement seems to be of an AAB type, *i.e.*, a composition of two identical and one different polypeptide chains. The native molecule has 11 total cysteine residues, each A subunit containing 5 of these. Four free sulfhydryl groups are easily titratable, whereas two more are assayable on prolonged incubations. When labeled with iodoacetamide, all free -SH groups are localized in one main peptide. (All free SH groups appear essential to activity.) The enzyme is dissociated into inactive dimers and monomers; however, monomer preparations can be reassociated to form active molecules of more than one type. Reassociation is dependent on pH, temperature, buffer, and sulfhydry reagents. Tartronic semialdehyde reductase (M. Wt. 104,000) has four subunits; hydroxypyruvate reductase (M. Wt. 72,000) has three. Both have similarly low total cysteine contents.

The tartrate dehydrogenase has been purified to homogeneity and crystallized. The 155,000 M. Wt. enzyme is composed of four subunits (M. Wt. 38,000). Complex requirements have been found for a divalent metal cation, monovalent metal cations, and ammonium ions. The latter cause an interesting dissociation of specificity, the meso-isomer oxidation being increased while the L-enantiomorph oxidation is inhibited.

(Drs. Leonard D. Kohn and William A. Warren)

Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

Studies have been carried out with purified enzymes which have pyridoxal phosphate as a cofactor and which carry out reactions in amino acid metabolism. The object of these studies has been to elucidate the mechanism of pyridoxal phosphate enzymes by determining the features of the proteins which influence their rates, specificities, and control. Spectrophotometric, optical rotatory dispersion, and chemical studies of tryptophan synthetase of *Escherichia coli* have demonstrated new enzyme-substrate intermediates and an unusual new thiol-dependent transamination reaction catalyzed by the B protein which produced two new sulfur-containing α-keto acids. S-pyruvylmercaptoethanol and S-pyruvyldithioerythritol. These studies have clarified intermediates and controls in the overall mechanism of this enzyme.

Studies on an enzyme complex with serine and the function of sulfhydryl groups in its formation promise to explain two of the roles of the A protein in this system.

The binding of pyridoxal phosphate and analogs to L-aspartate decarboxylase has also been studied by kinetic and optical methods.

(Drs. Edith Wilson Miles and Lindsay Sparrow)

Polyamine Metabolism and Function

Several aspects of polyamine metabolism and function have been under investigation, as follows.

(1) The significance and conditions for synthesis of the acetyl derivatives of the polyamines have been studied. These derivatives are formed *in vivo* only when cells are stored at low temperatures or chilled during harvesting, but are not present in *Escherichia coli* under normal growth conditions.

(2) Biosynthesis of spermidine and 1,4-diaminobutane in *Escherichia coli* are controlled by the levels of polyamines in the cells. Cells grown in medium containing polyamines take up amines; both inhibition and repression of ornithine decarboxylase occur resulting in a marked decrease in the formation of 1,4-diaminobutane from ornithine. Studies on the control of synthesis of polyamines from arginine via arginine decarboxylase are in progress.

(3) Further studies were carried out on the biosynthesis of spermidine. The enzyme carrying out the decarboxylation of adenosylmethionine has been purified a thousand-fold to homogeneity. The enzyme appears to have a pyridoxal phosphate cofactor.

(4) An enzyme in *Serratia marcescens*, which oxidizes spermidine, has been purified seven thousand-fold to homogeneity. This pure enzyme contains both a heme and flavin-adenine dinucleotide. Studies on the enzymatic mechanism of this unusual protein are in progress.

(5) Studies on the enzyme in *Escherichia coli* which carries out the formation of a spermidine-glutathione derivative have progressed. Large scale preparations of a fifty-fold purified enzyme can now be obtained. Further studies on the purification of this enzyme are planned.

(Drs. Herbert Tabor, Celia W. Tabor, and Reed B. Wickner, and
Mrs. Patricia D. Kellogg)

Purification and Characterization of Histidine Ammonia Lyase

Histidine ammonia lyase (histidase) has been purified to homogeneity from a pseudomonas (ATCC 11,299B) grown on histidine. Ultracentrifuge studies indicate a single molecular species with $s^{0}_{25,w}$ of 11.1 S and $D_{25,w}$ of 5.0×10^{-7} cm^{2} sec^{-1}. Amino acid analysis yields a $\bar{v}$ of 0.736 ml per g. A molecular weight of 214,000 is calculated from these data, in agreement with 211,000 from high speed sedimentation equilibrium. Disc gel electrophoresis at several pH's reveals only one protein species, coincident with enzymatic activity. Guanidine HCl and mercaptoethanol dissociate histidase into subunits of molecular weight 35,000, which give a single band on electrophoresis in 6 M urea.

Histidase loses activity on storage but can be reactivated with mercaptoethanol. Untreated, and ME-activated enzyme, differ strikingly in V_{max}, and sensitivity to EDTA and PCMB. The precise difference between these two enzyme states is not reflected in sedimentation coefficient or in titratable sulfhydryl groups (only 1-2 react with dithionitrobenzoate in urea or 20 total half-cystines).

(Drs. Matthew M. Rechler and Herbert Tabor)

Mechanism and Therapy of Acute and Late Deaths following Experimental Trauma

In the investigation of deaths after trauma, it was found that (1) vaccination by specific and non-specific pseudomonas vaccines, as well as therapy wit sulfadiazine and streptomycin, significantly protected burned mice against a topical pseudomonas challenge; (2) daily injection of a quantity of 0.85% NaCl equivalent to 15% of body weight significantly reduced the late mortality of burned mice; and (3) pre-treatment with purine compounds significantly protected mice against acute deaths after burn, tourniquet, and endotoxin shock. In the study of factors influencing swelling due to tourniquet trauma, a method was developed to measure swelling of the rodent tail; of the drugs tested in this model, only serotonin exerted a marked effect (inhibition) on swelling. In the search for agents to prolong the survival time of skin allografts, it was found that phytohemagglutinin, a mucoprotein from the red kidney bean, doubled the survival time of rabbit skin allografts.

(Drs. Kehl Markley and Sanford M. Rosenthal, and Mrs. Elizabeth T. Smallman)

Culture Studies on Mouse and Human Leprosy Bacteria

Streptomycin showed definite suppresive activity on the growth of *Mycobacterium lepraemurium* in macrophage cultures. Diaminodiphenylsulfone showed marked suppressive activity in murine leprosy in mice when very small numbers of organisms were innoculated in the foot pads of animals. The percentage of irregularly stained organisms increased both during the growth phase and death phase of *Mycobacterium lepraemurium* in macrophage cultures. Immunosuppressive drugs showed no effect on the growth of *Mycobacterium leprae* in macrophage cultures. Mice were injected with killed *Mycobacterium leprae* and imuran to induce a specific immunologic tolerance to *Mycobacterium leprae*. Macrophages obtained from these animals were then infected with *Mycobacterium leprae*. No sign of growth of the bacilli was observed.

(Dr. Yao Teh Chang and Mrs. Roxanna Lee Andersen)

Serial No. NIAMD-LBP-1
1. Biochemical Pharmacology
2. Biochemistry of Amino Acids
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry of Sulfur-containing compounds

Previous Serial Number: NIAMD-LBP-1

Principal Investigator: Simon Black

Other Investigators: Blondel Hazel, Lawrence Aronson

Cooperating Units: None

Man Years:

Total:	4-2/3
Professional:	4
Others:	2/3

Project Description:

Objectives: A long term objective is discovery of enzymatic mechanisms involved in the synthesis of constituents of living tissue, particularly of proteins.

Methods employed: Chemical and enzymatic techniques involving the use of spectrophotometry and radioactive substances are evolved to meet the needs of special problems as they arise.

Major findings: An enzyme has been isolated from yeast and purified to homogeneity, which catalyzes the following reaction:

$$2\ GSH + RSSR \longrightarrow GSSG + 2\ RSH$$

The molecular wright is estimated to be about 15000. An investigation of its kinetic behavior has revealed that the enzyme has a higher affinity and a more rapid reaction rate with L-cystine than with any other disulfide tested, including D-cystine, L-cysteine diamide, L-cystinyl diglycine, L-homocystine, β-hydroxyethyl disulfide, and several disulfide containing hormones and proteins.

These results suggest that the function of the enzyme *in vivo*, or one of its functions, may in fact be the reduction of cystine, which tends to form from cysteine by nonenzymatic oxidation. The question arises whether a specific agent is required for this purpose, inasmuch as nonenzymatic reduction by GSH should be expected to occur at a substantial rate. The existence of the disease called cystinosis in humans, in which cystine does accumulate in cells despite a normally functioning glutathione reductase system, suggests that there may be a definite need for a cystine-reducing enzyme. It appears that no previous evidence to support such a specific reduction has been reported.

Significance to Bio-medical Research and the Program of the Institute: The interchange of S-S and -SH bonds is an important general process in cells. One possible relation to disease processes is apparent from the disease cystanosis which could arise from a defect in such an interchange mechanism.

Proposed Course of the Project: Work on the mechanism of the enzyme catalyzed -SH, S-S interchange reaction will continue with special attention to a possible role in rate regulating processes. Work on rate controlling aspects of the t-RNA aminoacylating enzymes is also in progress.

Honors and Awards: None.

Publications: Nagai, S., and Black, S., J. Biol. Chem. 243: 1942, (1968).

Serial No. NIAMD-LBP-2
1. Biochemical Pharmacology
2. Biochemistry of Amino Acids
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Chemotherapy of Mouse Leprosy
Previous Serial Number: Same
Principal Investigator: Y. T. Chang

Cooperating Units: Leonard Wood Memorial (The American Leprosy Foundation).

Man Years:

Total: 3
Professional: 2
Others: 1

Project Description:

Objectives: The evaluation of therapeutic effectiveness of drugs in mouse leprosy. The tissue cultures of intracellular parasites.

Major Findings: Serial transfers of M. lepraemurium in macrophage cultures have been continued for a total of 38 transfers in 171 weeks in one series and 35 transfers in 155 weeks in another. The cumulative increase of organisms per macrophage was 5×10^{45}-fold in the former and 3.4×10^{45}-fold in the latter.

Streptomycin, 0.5 to 100 μg/ml showed definite suppressive activity intracellularly on the growth of M. lepraemurium. This has been a much debated question among many laboratories during the past 20 years. The confusing factor has been the simultaneous extracellular growth of organisms in the culture. Our M. lepraemurium-macrophage system offers a special advantage in clarifying this question, since M. lepraemurium grows exclusively intracellularly.

Morphological studies on the growth of M. lepraemurium in cultures of macrophages revealed an increase in the percentage of irregularly stained organisms in both growth phase and death phase of the bacilli. The difference was that bacillary elongation and multiplication were observed in the growth phase but not in the death phase. This is important since presently irregularly stained organisms are considered dead by many researchers in both experimental and human leprosy.

DDS showed marked suppressive activity in murine leprosy in mice when very small number of organisms (5×10^{3}) were inoculated in the foot pad of animals. Even a dose level as small as 0.00001% in the diet was suppressive. This is quite different from observations in animals infected intraperitoneally with a huge inoculum containing 10^{9} organisms. Difference in DDS activity between the two schedules depends chiefly on the size of inoculum used. This is of paramount interest, since a similar activity of DDS in the mouse foot-pad infection of human leprosy bacilli has been considered as characteristic both for the drug and for the infection.

Difficulty has been met recently in obtaining commercial beef embryo extract free of antibiotics. Chick embryo extract was found to be satisfactory for maintenance of macrophages in cultures, but the acid-fast rods (rods and cones of the retina of eyes of embryos), which accumulated in macrophages after frequent renewal of medium simulated an appearance of growth of acid-fast bacilli. Filtering chick embryo extract eliminated the rods but was time consuming and the appearance of macrophage was not very good on prolonged cultures. A special chick embryo extract made of embryos freed of eyes before processing was found satisfactory and is available commercially.

Studies of the effect of the immunosuppressive drugs, imuran and cortisol, on the growth of M. leprae in macrophage cultures revealed negative results. Mice were injected with a large number of killed M.leprae ($12.5x10^6$) intravenously. These same animals were then injected daily with imuran, 1 mg. per mouse subcutaneously, 5 days a week, for one month in order to induce a specific immunologic tolerance to M. leprae. Macrophages obtained from these animals were then infected with organisms from a fresh biopsy of a lepromatous patients. No sign of growth of M. leprae was observed after a period of 15 weeks in the culture.

In cooperation with Dr. A. P. Monoco of Harvard Medical School and Dr. J. N. Sheagren of NIAID, inoculation of M. leprae was made in foot pads of mice which had been thymectomized and receiving anti(mouse)-lymphocyte serum in order to observe the process of desimination of organisms to the whole animal instead of localized growth in the foot pads as observed in conventional animals. The experiment is in progress.

Arrangement for a regular supply of biopsy from leprosy patients from the Provincial Lo-Shing Leprosarium of Taiwan has been achieved. Five biopsy shipments have been received last year.

Honors and Awards: None.

Publications: None.

Serial No. NIAMD-LBP-3
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism and Therapy of Acute and Late Deaths Following Experimental Trauma.

Previous Serial Number: NIAMD-LBP-3

Principal Investigator: Kehl Markley

Other Investigators: Sanford M. Rosenthal, Elizabeth Smallman

Man Years (computed for the 12-month period):

Total:	5-1/4
Professional:	3
Other:	2-1/4

Project Description:

Objectives: (1) To investigate the prophylaxis of pseudomonas infection in burned mice by vaccination; (2) to observe the effect of pre-treatment with purine compounds in mice after burn, tourniquet and endotoxin shock; (3) to determine the role of fluids, ambient temperature and diet on the delayed deaths of burned mice; (4) to study the factors that influence swelling produced by tourniquet trauma; and (5) to discover agents that prolong the survival time of skin allografts of inbred strains of mice.

Methods employed: (1) Vaccines were prepared from cells and media of the 10 serologic types of P. aeruginosa isolated by Verder. Mice were vaccinated prior to or after thermal trauma and challenged at various time intervals with P. aeruginosa. Cross protection experiments were performed. Mortality was recorded for 21 days post-burn. In addition a comparative survey of chemotherapeutic agents was made in the treatment of pseudomonas septicemia in mice. (2) Many purine and pyrimidine compounds, as well as related compounds, were injected ip. and sc. at various time intervals before or after the production of burn, tourniquet and endotoxin shock. Dosage of these compounds varied from 10-80 μmoles/mouse. Forty-eight hour mortality was used to test the efficacy of these materials. (3) Mice were given a 2/3 body surface area burn in water at 70°C for 8 seconds. The effect of fluids in late mortality was tested by giving daily s.c. injections of 5%, 10%, 15% and 20% of body weight of 0.85% NaCl or 5% glucose for 21 days. The effect of diet in late mortality was studied by feeding weanling mice on diets containing various amounts of casein protein prior to thermal trauma. Mortality was recorded for 21 days post-burn. The effect of ambient temperature was investigated by keeping germfree mice at 25°C and 31°C prior to and after the burn described above. Mortality was recorded for 21 days post-burn. (4) A method was devised for measuring the volume of the rodent tail by the amount of fluid displaced from one arm of a U-tube manometer. The effects of standardized trauma by tourniquets or burns were studied. Both acute and chronic swelling could be produced by varying the length of the ischemia. The effects of adrenalin, nor-adrenalin, histamine and serotonin injected ip. on acute swelling were measured. (5) The effect of the administration of phytohemagglutin, a mucoprotein extracted from the red kidney bean, on survival time of skin allografts between BALB/c and DBA/2 mice was studied.

Major findings: (1) Vaccination by specific and non-specific pseudomonas vaccines, as well as therapy with sulfadiazine and streptomycin, significantly protected burned mice against a topical pseudomonas challenge. (2) Daily injection of a quantity of 0.85% NaCl equivalent to 15% of body weight significantly reduced the late mortality of burned mice. (3) Pretreatment with purine compounds significantly protected mice against acute deaths after burn, tourniquet and endotoxin shock. (4) A method was developed to measure swelling of the rodent tail after tourniquet trauma. Of the drugs tested in this model, only serotonin exerted a marked effect (inhibition) on swelling.

Proposed course of action: To continue studies related to: (1) the role of endotoxin after traumatic shock; (2) the therapy of pseudomonas infection in burned mice; (3) the factors involved in swelling after tourniquet trauma; (4) the mechanism of action of purines in protection against burn, tourniquet and endotoxin shock, and (5) agents that prolong survival time of skin allografts in inbred strains of mice.

Honors and Awards: None.

Publications:

1. Markley, K., Smallman, E. and Evans, G., Decrease of mouse circulating antibodies to sheep erythrocytes by phytohemagglutinin. Int. Arch. Allergy and Applied Immunol. 32: 482, (1967).

2. Markley, K., Evans, G. and Smallman, E., Prolongation of Skin allograft survival time by phytohemagglutinin. Transplantation, 5: 1535, (1967).

Serial No. NIAMD-LBP-4
1. Biochemical Pharmacology
2. Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry of Polysaccharides and Glycoproteins

Previous Serial Number: Same

Principal Investigator: Dr. Victor Ginsburg

Other Investigators: Dr. Laura Shen, Dr. Barbara Scher, Dr. Roger Winand, Dr. Evelyn Grollman, and Dr. Akira Kobata.

Man Years:

Total:	7½
Professional:	5½
Other:	2

Project Description:

Objectives:

To investigate the synthesis and function of polysaccharides and glycoproteins.

Methods Employed:

Usual biochemical techniques.

Major Findings:

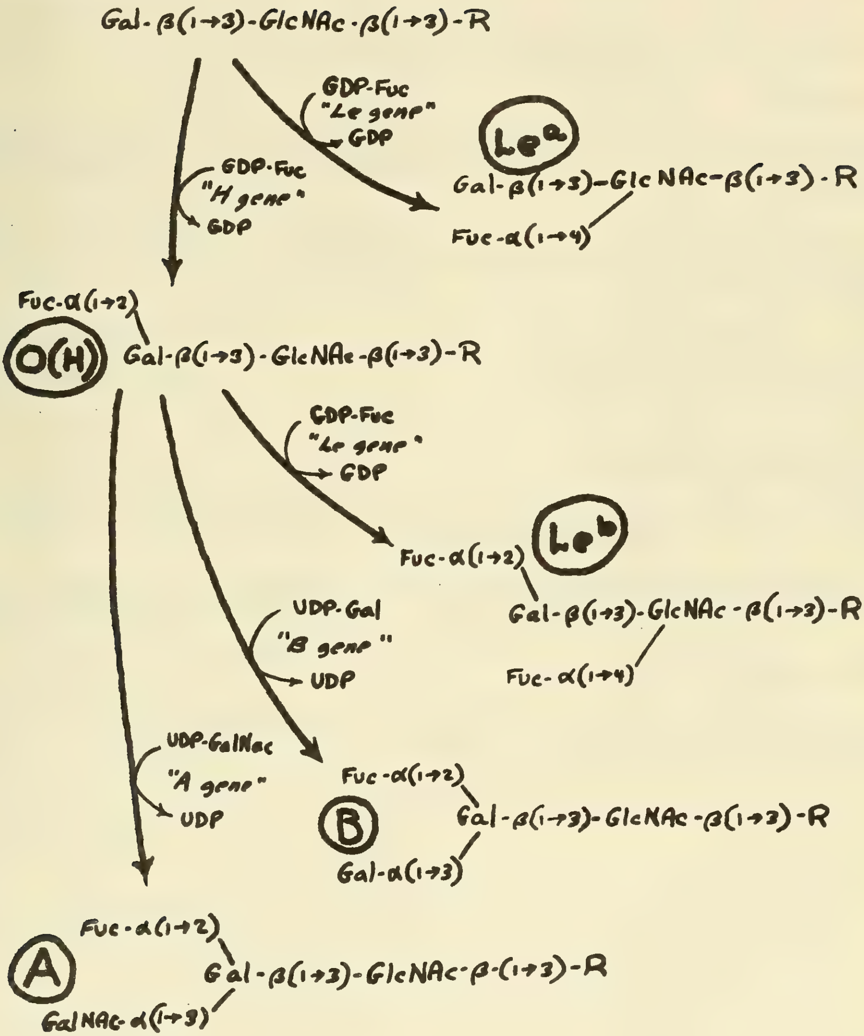
Injection of TSH to guinea pigs produces (1) an increase in $^{35}SO_4$ uptake by harderian glands (increased sulfate is mainly in the dialysable fractions after a single TSH injection, but there is also increased sulfate in the mucopolysaccharide fraction if TSH is injected daily for 3 and 5 days); (2) an accumulation of mucopolysaccharides by harderian gland which is maximal after 3 and 5 days of daily injections but disappear after 15 days; (3) a modification in wet weight which parallels the level of mucopolysaccharide and the appearance and disappearance of visible exophthalmos; (4) an increase in $^{35}SO_4$ uptake and mucopolysaccharides content in the perirenal fatty tissue and intestine but not in the skin and muscle. All the mucopolysaccharides normally present in harderian gland are proportionally increased after TSH injection.

Major Findings (continued):

The excretion of mucopolysaccharides normally found in urine (chondroitin, chondroitin sulfate A and C, keratosulfate, and heparitin sulfate) is increased approximately twofold in patients with progressive exophthalmos. A threefold elevation of total serum mucopolysaccharides is also found. These increases are unrelated to thyroid function.

Differences in the structures of carbohydrates which occur on the surface of red blood cells and in secretions such as saliva and milk are responsible for differences in certain blood types that distinguish humans. It has been found that the presence or absence of specific transferases in individuals is genetically controlled and determines the structure of these complex carbohydrates and hence the blood type of the individual. Therefore, unlike proteins whose structure is directly determined by genes and are primary gene products, the structural determinants of blood type can be considered to be secondary gene products. That is to say, the direct products of the genes that determine blood type are enzymes and it is these enzymes working in concert that are responsible for the formation of the specific antigenic structures. This type of mechanism explains the well-known fact that individuals can have cell surface antigens not possessed by either parent and provides a possible mechanism for the expression of individuality. The four transferases that have been demonstrated thus far are summarized below. The presence or absence of these enzymes determines six blood group specificities - type XIV, Le^{a}, Le^{b}, O(H), A and B (shown circled next to their respective structural determinants). The "Le" enzyme is found in individuals with blood type Le^{a} or Le^{b} and is absent in Le(a-b-) individuals. The "H" enzyme is present in individuals who elaborate O(H), A, B or Le^{b} substances ("secretors") and is absent in those who do not ("nonsecretors"). The "A" enzyme is found in individuals with blood type A or AB and not in those with blood type B or O while the "B" enzyme is found in individuals with blood type B or AB and not in those with blood type A or O.

TYPE XIV



Transglycosylases of Humans.

R = lactose

Significance to NIAMD Research:

These studies contribute to an understanding of the biochemistry of polysaccharides and glycoproteins.

Publications:

Shen, L., and Ginsburg, V.: Sugar Analysis of Cells in Culture; Determination of the Sugars of HeLa Cells by Isotope Dilution. Arch. Biochem. Biophys. 122: 474-480, Nov., 1967.

Grollman, E. F., and Ginsburg, V.: Correlation Between Secretor Status and the Occurrence of 2'-Fucosyllactose in Human Milk. Biochem. Biophys. Res. Commun. 28: 50-53, 1967.

Shen, L., Grollman, E. F., and Ginsburg, V.: An Enzymatic Basis for Secretor Status and Blood Group Substance Specificity in Humans. Proc. Natl. Acad. Sci. 59: 224-230, Jan. 1968.

Kaufman, R. L., and Ginsburg, V.: The Metabolism of L-fucose by HeLa Cells. Experimental Cell Research 50: 1-7, 1968.

Kobata, A., Grollman, E. F., and Ginsburg, V.: An Enzymic Basis for Blood Type A in Humans. Arch. Biochem. Biophys. 124: 609-612, 1968.

Scher, B. M., and Ginsburg, V.: Isolation of Adenosine 5'-Diphosphate D-Mannitol from Salmonella typhimurium. J. Biol. Chem. 243: 2385-2391, 1968.

Shen, L., and Ginsburg, V.: Release of Sugars from HeLa Cells by Trypsin. Wistar Monograph 8: 16-21, 1968.

R. Winand: Increased Urinary Excretion of Acidic Mucopolysaccharides in Exophthalmos. J. Clin. Invest., in press.

Serial No. NIAMD-LBP-5
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Estimation, Metabolism, and Function of Amines

Previous Serial Number: NIAMD-LBP-5

Principal Investigator: Herbert Tabor

Other Investigators: Celia W. Tabor, Patricia Kellogg, Elizabeth Sweeney, and Reed Wickner

Cooperating Units: None

Man Years

Total: 6
Professional: 4-1/2
Other: 1-1/2

Project Description:

Objectives: The importance of the various polyamines in shown by their wide distribution in viruses, bacteria, plant and animal cells. Studies are being conducted to elucidate their metabolism and function.

Methods Employed: The amines and their derivatives are studied by isotopic labeling and special chromatographic techniques. Standard enzymological techniques have been used to purify further the enzymes that degrade spermine, spermidine, and monoamines. Chemical synthetic procedures have been utilized to prepare derivatives for identification of enzymatic products. Uptake of amines was studied by counting the residual radioactivity in washed pellets on Millipore filters. Spectrophotometric methods have been used to assay serum amine oxidases.

Major Findings: The enzyme carrying out the oxidation of spermidine in *Serratia marcescens* has been purified 7000-fold to homogeneity. The pure enzyme has a molecular weight of 75,000. Both a heme and flavin have been identified in the pure protein. Approximately 1 mole of heme and 1 mole of flavin-adenine dinucleotide are present per mole of enzyme. The heme moiety has been shown to be protoporphyrin IX. The enzyme in *E. coli* which carries out the synthesis of the spermidine-glutathione derivative has been partially purified on a large scale, achieving about a 50-fold purification. This enzyme has been very unstable during these large-scale purifications, but has been stabilized with dithioerythritol.

The monoacetylspermidine derivatives and monoacetyl-1,4-diaminobutane have been described in E. coli both in our laboratory and by others. However the acetyl derivatives are not found in cells which are grown at 37° in minimal medium and harvested rapidly at room temperature. The formation of the acetyl derivatives of the polyamines occurs only upon chilling the bacterial culture before harvesting the cells. If cells are maintained at 5°-10° for 2-3 hours, as much as 60% of the intracellular spermidine may be converted to monoacetylspermidine. Acetylation of 1,4-diaminobutane (putsescine) may be as high as 10% of the total diamine. The striking alterations in polyamine metabolism on cooling cultures may be only a manifestation of alterations in the pool of other cell components, such as nucleic acids, or other acids, or may reflect a cold-activated enzyme, or an increased pool of acetyl Co A.

The decarboxylation of s-adenosylmethionine is the second of 3 steps in the biosynthetic pathway leading to the formation of spermidine from methionine. The constitutive enzyme which carries out this reaction in wild-type E. coli has been purified 1000-fold. The steps consist of an ammonium sulfate fractionation, a heat step, chromatography on DEAE-cellulose and DEAE-Sephadex, and preparative acrylamide disc gel electrophoresis. The purest preparation is homogeneous on analytical disc gel electrophoresis. Preliminary studies suggest that pyridoxal phosphate may be the cofactor involved in this reaction.

The control of biosynthesis of the amines in an ornithine-requiring mutant of E. coli, have been carried out using a chemostat to limit the available ornithine. ^{14}COOH-labeled ornithine has been used in these studies. The formation of total amines was followed by the liberation of $^{14}CO_2$. Inhibition and repression of the ornithine decarboxylase occur in the presence of 1,4-diaminobutane and spermidine. Studies on the control of polyamine biosynthesis via arginine decarboxylation are in progress.

There have been several reports which have identified polyamines as the physiological agents controlling RNA synthesis. These conclusions were drawn from experiments with an amino acid-requiring mutant of E. coli (15TAU$^-$) in which RNA synthesis is under stringent control. This organism makes almost no RNA when the essential amino acid is missing from an otherwise adequate medium. However, in the presence of the required amino acid, RNA is synthesized normally. In this organism, starved of its amino acid, Raina and Cohen found that very high concentrations of spermidine stimulated RNA synthesis. This effect has been confirmed; however, this extremely high concentration of spermidine strikingly inhibits protein synthesis, and even stimulates protein degradation. Consequently the polyamine-stimulated-RNA synthesis can be attributed to an effect comparable to that observed with chloramphenicol or other inhibitors of protein synthesis. Thus far, there is no evidence for any direct control of RNA synthesis by polyamines.

Significance to Bio-medical Research and the Program of the Institute: Recent work has indicated that the polyamines play an important role in permeability of organisms and in the stability of nucleic scids inside cells. The importance of the amines in cell physiology is suggested even more strongly by our recent findings that energy-dependent uptake systems exist for these amines.

The polyamines in the cell remain as 1,4-diaminobutane or spermidine; there is no turnover or degradation in a growing culture in minimal medium. We now find that acetylation of polyamines which we and others have previously reported is secondary to the very general practice of cooling a culture to 0°, and centrifuging the cells in the cold in order to prevent alterations in cell contents during harvesting. A progressive conversion of both spermidine and, to a lesser extent, of 1,4-diaminobutane to the monacetylated forms occurs with time during storage at 5-10°. These observations may be indicative of other widespread alterations which occur during chilling. The effect of the conditions under which cells are harvested will have to be carefully evaluated for the particular systems being studied.

The synthesis of polyamines from ornithine in E. coli appears to be under strict control; both inhibition of the constitutive ornithine decarboxylase and repression of this enzyme formation occur with added spermidine or 1,4-diaminobutane.

A pure enzyme, spermidine dehydrogenase, isolated from Serratia marcescens, contains a mole of heme and a mole of FAD per mole of protein. This enzyme will be a very useful protein for the study of electron transport mechanisms.

Studies on the spermidine-glutathione synthetase and the adenosylmethionine decarboxylase will help determine the mechanisms by which these reactions are carried out.

Proposed Course of the Project: Further studies on the metabolism and function of the biologically important amines. We hope to learn the normal physiological function of these amines in the cell, and to explain such pathological effects as the production of renal damage.

Honors and Awards: None

Publications:

Tabor, C. W.: The Effect of Temperature on the Acetylation of Spermidine. Biochem. Biophys. Res. Commun. 30:339 (1968).

Serial No. NIAMD-LBP-6
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzymatic Studies of Nucleic Acid Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. Nancy G. Nossal

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives:

To determine the characteristics and functions of enzymes induced by T_{even} phage which are involved in the synthesis, modification, and degradation of DNA.

Methods Employed:

Standard biochemical techniques were employed for the preparation of polynucleotide substrates and enzymes. Extensive use has been made of amber mutants of T_4 bacteriophage which are unable to make specific early enzymes in nonpermissive hosts. A spectrophotometric assay for the hydrolysis of short oligonucleotides has been developed.

Major Findings:

It has been shown previously in this and other laboratories that after T_{even} bacteriophage infection there are at least two new, physically separable, enzyme fractions which are able to degrade denatured DNA. One of these DNases is physically inseparable from the phage induced DNA polymerase. We have now shown that the two DNases can be distinguished on the basis at the relative rates of hydrolysis of substrates of different sizes. The polymerase-associated nuclease degrades both DNA which has been digested to a limited extent by pancreatic DNase and very short oligonucleotides at similar rates, while the second enzyme (termed oligonucleotide diesterase) hydrolyzes the short chains very much faster than the longer ones. Deoxynucleotide triphosphates, which inhibit the first nuclease, have no effect on the second.

Several amber mutants of T_4 have been examined for changes in nuclease and polymerase activity. Most of the mutants in gene 43, which has been shown by others to code for the DNA polymerase, lack both the polymerase and the polymerase-associated nuclease. The oligonucleotide diesterase is present at normal levels in all gene 43 mutants, showing that the two nucleases are the products of different genes. One mutant in gene 43 has been found which lacks the polymerase but has present what appears to be a somewhat modified polymerase-associated nuclease. It differs from the wild type enzyme in its chromatographic properties and substrate specificity. Amber mutants in genes 46 and 47, which have been previously shown by others to be unable to degrade the host DNA, have normal levels of both the nucleases studied here, suggesting that other enzymes are required for the degradation of the bacterial DNA.

Significance to the Program of the Institute:

Knowledge of enzymes controlling DNA synthesis and degradation is important to an understanding of the control of DNA replication and transcription in both normal and viral infected cells.

Proposed Course:

Other mutants with modified DNA polymerases and polymerase-associated nucleases will be sought and the enzymes produced by these mutants purified and studied to determine what structural changes are responsible for the changes in the enzymatic properties of the proteins. The basis for the substrate specificity and the function of these nucleases in the infection process will be investigated, and the search for the enzyme(s) controlled by genes 46 and 47 will be pursued.

Honors and Awards:

None.

Publications:

Nossal, N. G., and Singer, M. F.: The Processive Degradation of Individual Polyribonucleotide Chains. I. *Escherichia coli* Ribonuclease II. J. Biol. Chem. 243: 913-922, March 10, 1968.

Serial No. NIAMD-LBP-7
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Ribosomal Structure and Function

Previous Serial Number: Same

Principal Investigator: Anthony V. Furano

Other Investigator: Vincent W. Hollis, Jr.

Cooperating Unit: None

Man Years:

Total: 2 1/2
Professional: 2
Other: 1/2

Project Description:

Objectives:

To study the relationship between ribosomal structure and function.

Methods Employed:

Two lines of approach are being used. The first consists of investigating the functional capacities of ribosomes that have been modified either by treatment with a selective chemical reagent or by genetic alteration. The modified ribosomes are tested for their ability to bind messenger RNA (*e.g.*, poly U) and aminoacyl s-RNA, and to participate in polypeptide synthesis *in vitro*. Identification of the ribosomal modification allows one to couple the functional defect of the ribosome with the chemical and biological nature of the ribosome.

The second line of approach consists of studying the soluble enzymes that interact with ribosomes and catalyze in some way polypeptide synthesis. A thorough investigation of the properties and characteristics of such enzymes is a prerequisite to any understanding of interaction with ribosomes and their role in protein synthesis. Accordingly, work is underway to characterize the stable and highly purified preparation of these enzymes that we have been able to achieve.

Major Findings:

(1) Chemical studies on highly purified ribosomes.

We had reported preliminary findings (Furano and Hollis, *Fed. Proc.*, 1967) that mercaptoethanol inactivated ribosomes by causing a specific defect in the 30S ribosomal subunit. Further work indicated that this mercaptan effect on ribosomes was rather complex and that mercaptoethanol may have more than one effect on ribosomes. At least one of the deleterious effects of mercaptoethanol was dependent on O_2. We subsequently showed that this O_2 dependent effect of mercaptoethanol was probably traceable to the evolution of oxidized mercaptoethanol (diethanoldisulfide) during the aerobic preincubation of the ribosomes with mercaptoethanol. Thus we were able to show that diethanoldisulfide under anaerobic conditions inactivated ribosomes in a selective fashion such that the ribosomes could no longer bind aminoacyl tRNA but could still bind messenger RNA. In this study we also showed that aerobiosis itself during storage is deleterious to ribosomes and that anaerobic storage of ribosomes prolonged their life from less than 3 weeks to more than 4 months.

(2) Isolation of mutants of *E. coli* with temperature sensitive ribosomes.

This work is currently in progress. We have been able to isolate a number of temperature sensitive mutants of *E. coli* that were selected simultaneously for streptomycin resistance as well as temperature sensitivity. Since the mutation to streptomycin resistance may result in altered ribosomes the chances may be fairly good of having selected in this manner mutants whose altered ribosomes are temperature sensitive. We are now planning to characterize the ribosomes of such mutants.

(3) Nature of the soluble factors necessary for ribosomal dependent polypeptide synthesis.

We have been able to devise a new method for purifying the soluble factors necessary for ribosomal dependent polypeptide synthesis. The main features of this work have shown the following.

(a) The previously described factors {T (Ts and Tu) and G} may be derived from (perhaps proteolytic products) a substance that *by itself* is able to catalyze polypeptide synthesis with highly purified ribosomes.

(b) We have been able to stabilize and purify this substance, which we have called E-1, 200-fold.

(c) During purification we separated from E-1 a low molecular weight substance, which we have called E-2, which is also required for polypeptide synthesis.

(d) E-1 and E-2 are sufficient to catalyze polypeptide synthesis with highly purified ribosomes.

(e) E-1 and E-2 are both very stable. We are currently investigating the role of these two factors in polypeptide synthesis.

We have reported these findings at the Federation meetings in April (Furano and Hollis, *Fed. Proc.,* 1968) and have submitted a report of them for publication in the *Proceedings of the National Academy of Sciences.*

Significance to the Program of the Institute:

Knowledge on the relationship between ribosomal structure and function will aid in the understanding of some of the basic steps in protein synthesis.

Proposed Course:

This work is currently in progress and will be continued and extended.

Honors and Awards:

None.

Publications:

Furano, A. V.: Selective Loss of Binding of Aminoacyl tRNA by Ribosomes Treated with Diethanoldisulfide. Biochim. Biophys. Acta. (In Press)

Serial No. NIAMD-LBP-8a
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Inhibition of Bacteriophage T_4 Maturation by Actinomycin D

Previous Serial Number: Same

Principal Investigator: Dr. David Korn

Other Investigators: Miss Kathy McDonald (Summer Youth Opportunity Employee), 3 months

Cooperating Units: None

Man Years

Total: 1 1/4
Professional: 1
Others: 1/4

Project Description:

Objectives:

To elucidate the mechanism by which actinomycin D selectively blocks the maturation of phage T_4.

Major Findings:

In continuation of our studies of the mechanism of selective inhibition of bacteriophage T_4 maturation by actinomycin D, we have isolated a number of T_4 mutants that are partially resistant to this drug. In conjunction with a summer employee, Miss K. MacDonald, we have mapped four of these mutants in a single cistron that is known from the work of Edgar *et al.* to be concerned with the assembly of phage components and the packaging of phage "replicating" DNA within phage heads. Further work with these mutants will be directed toward attempting to define the mechanism of DNA packaging and the function of this "assembly locus."

We have also studied the effect of a large number of actinomycin congeners and have found, in general, that all of the compounds previously shown to be inactive in the inhibition of RNA polymerase and unable to bind to DNA are inactive against T_4 maturation. However, two compounds, representing the chromophore moiety of the actinomycin molecule are slightly effective in the phage system. These compounds, neither of which appears to bind to DNA, are phenoxazinone and phenoxazinone-1,9-dicarboxylate methyl ester. Further study of these compounds should prove helpful in elucidating the mechanism by which actinomycin appears selectively to block phage DNA packaging into the phage head.

Significance to the Program of the Institute:

This system provides a model for the understanding of the morphogenesis of an infectious viral particle and the inhibitory effect of drugs thereon. This is an area of great potential significance to the development of a rational anti-viral chemotherapy.

Honors and Awards:

Maryland Academy of Science's "Distinguished Young Scientist" Award

Publications:

Korn, David, Gelderman, Albert, Cage, Gary, Nathanson, Donald, and Strauss, Arthur J. L.: Immune Deficiencies, Aplastic Anemia and Abnormalities of Lymphoid Tissue in Thymoma. New Eng. J. of Med. 276: 1333-1339, June 15, 1967.

Greene, R., and Korn, D.: The Instability of T_4 Messenger RNA. J. Mol. Biol. 28: 435-443, 1967.

Yarmolinsky, M., and Korn, D.: Evidence for Independent Segregation of the *Escherichia coli* Chromosome and Non-replicating Bacteriophage λb2. J. Mol. Biol. 32: 475-479, March 1968.

Serial No. NIAMD-LBP-8b
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: DNA Polymerase from a Human Cell Line

Previous Serial Number: None

Principal Investigator: Dr. David Korn

Other Investigator: Dr. Richard Greene

Cooperating Units: None

Man Years

Total: 1
Professional: 1
Others: 0

Project Description:

Objectives:

To purify and characterize the DNA polymerase from KB cells.

Major Findings:

We are purifying and characterizing the DNA polymerase from a mammalian cell system, KB cells, as an initial approach to the problem of adenovirus replication in these cells. The problem has been complicated by the extreme lability of the partially purified enzyme, but we have now devised a procedure that yields polymerase in good yield at about a thousand-fold apparent purification. The purified preparation contains only a single major protein band by analytical disc gel electrophoresis. We are proceeding to characterize this enzyme.

Significance to the Program of the Institute:

The KB cell is a common host used for studies of numerous DNA viruses. The definition of the normal enzymes of nucleic acid metabolism in these cells is a necessary beginning toward the understanding of the changes caused by virus infection.

Publications:

None.

Serial No. NIAMD-LBP-8c
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effect of Actinomycin D on Bacteriophage Lambda Development in *Escherichia coli*

Previous Serial Number: Same

Principal Investigator: Dr. David Korn

Other Investigators: Dr. Nancy Lee Couse (3 months) and Dr. Doris Dahl, Visiting Associate (9 months)

Cooperating Units: None

Man Years:

Total: 2
Professional: 2
Others: 0

Project Description:

Objectives:

To extend our studies of the blockage of phage maturation by actinomycin D to a lambda system.

Major Findings:

We have continued the investigation begun a year earlier with Dr. N. L. Couse into the mechanism of λ DNA replication. Under the conditions of λ infection of *Escherichia coli*, pulse-labeled phage DNA is initially isolated as a very rapidly sedimenting complex with $S > 1{,}000$. Techniques have been developed for releasing the DNA from the complex. Results thus far indicate that the isolated pulse-labeled λ DNA sediments together with purified λ marker DNA in both neutral and alkaline sucrose gradients. Thus we find no evidence of closed circular DNA molecules or of DNA "concatenates." The data also indicate that some of the "unusual" DNA forms that have been described by other workers may be artifacts caused by the lysis and extraction methods employed.

Significance to the Program of the Institute:

This experimental system should be helpful in elucidating the mechanism of temperate phage maturation and in determining the significance of the various molecular forms of lambda DNA that have been described in phage-infected cells.

Publications:

None.

Serial No. NIAMD-LBP-9
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda

NIH-PHS
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Chemical and Physiological Alteration in the Surface of *Escherichia coli*
Previous Serial Number: Same
Principal Investigator: Dr. Loretta L. Leive

Other Investigators: Dr. Stuart B. Levy and Dr. Mary Jane Voll

Cooperating Unit: Dr. Stephan E. Mergenhagen of the National Institute of Dental Research

Man Years:

Total: 3
Professional: 2 1/2
Other: 1/2

Project Description:

Objectives:

To study surface changes in *Escherichia coli* produced by EDTA.

Major Findings:

It was previously found that treatment of *Escherichia coli* with ethylenediaminetetraacetate results in a nonspecific increase in permeability and release of surface components including half of the surface lipopolysaccharide.

The lipopolysaccharide (LPS) released by EDTA treatment has now been further characterized. In collaboration with Dr. Stephan E. Mergenhagen of the National Institute of Dental Research, it has been shown to be as antigenic and toxic as LPS extracted from the whole cell by conventional means. The released LPS can be separated into two fractions by ultracentrifugation. One fraction differs in composition from the rest of the cell's LPS by having a higher content of antigenic side chains. The second fraction has a normal LPS composition but contains lipid and protein as well as lipopolysaccharide. Removal of the lipid alters its sedimentation properties. It is possible that these molecules are released in association because they occur together in a surface structure.

In other experiments, LPS was labeled for various lengths of time. It was found that newly synthesized LPS is not removed by EDTA treatment while older

LPS is partially removed. This result indicates a metabolic difference between removed and retained LPS.

To determine whether there is any connection between the release of cell surface components and the permeability change, we have been isolating mutants that (a) show increased permeability without EDTA treatment and (b) are resistant to the EDTA-caused permeability change. The cell surface composition of such mutants will be compared with the wild type, both before and after EDTA treatment.

Significance to the Program of the Institute:

The action of EDTA on the cell surface allows the entry of many agents normally excluded and may have practical implications for drug therapy. Dissection of the cell surface by EDTA treatment will aid in understanding its structure, which will in turn help elucidate how the bacterium normally interacts with its environment.

Proposed Course:

Further studies on characterization of the material released by EDTA, especially the protein and phospholipid moieties, are planned. The metabolic relationship between released and retained material will be further studied by specific labeling. The newly-isolated mutants described above will be studied to determine which chemical changes are responsible for the permeability increase.

Honors and Awards:

None.

Publications:

Leive, Loretta: Studies on the Permeability Change Produced in Coliform Bacteria by Ethylenediaminetetraacetate. The Jour. of Biol. Chem. 243: 2373-2380, May 10, 1968.

Serial No. NIAMD-LBP-10
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

Previous Serial Number: Same

Principal Investigator: Dr. Edith Wilson Miles

Other Investigators: Dr. Lindsay Sparrow

Cooperating Units: Dr. I. P. Crawford of the Scripps Clinic and Research Foundation, La Jolla, California

Man Years:

Total: 2
Professional: 1 3/4
Other: 1/4

Project Description:

Objectives:

To gain further understanding of features of enzymes which contribute to their conformation, subunit interaction, and mechanism of action. Pyridoxal phosphate enzymes have been chosen for study because they are important in amino acid metabolism and because pyridoxal phosphate has optical properties which make it a useful probe of the active site of the enzymes to which it is bound.

Methods Employed:

Optical studies (spectroscopy, optical rotatory dispersion, circular dichroism, and fluorimetry) are carried out on highly purified pyridoxal phosphate enzymes (L-aspartate decarboxylase, L-tryptophan synthetase, and glutamic-oxaloacetic transaminase) to determine the conformation and reactions of enzyme-bound pyridoxal phosphate and pyridoxal phosphate-substrate intermediates. Kinetic studies are carried out to determine factors involved in pyridoxal phosphate binding and the rates and specificities of reactions. Studies with isotopes are carried out to determine new reaction products and enzyme-bound intermediates. Chemical modification studies are carried out to determine the role of specific functional groups at the active sites of the enzymes. New substrates and substrate or cofactor analogs are synthesized.

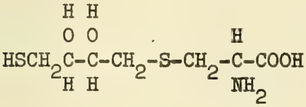
Major Findings:

(1) Tryptophan synthetase of *Escherichia coli* has been shown by other workers to be a complex enzyme composed of two different proteins, the A and B proteins, which carry out a variety of reactions depending on several controlling conditions. In order to clarify the mechanism and controls of these reactions, optical studies of the pyridoxal phosphate-containing B protein in the presence and absence of the A protein, substrates, and thiols have been carried out. The B protein has been discovered to carry out two new thiol-dependent reactions 1 and 2.

$$\{1\}\quad RSH + Serine \xrightarrow{PLP} R\text{-}S\text{-}Cysteine + H_2O$$

$$\{2\}\quad RSH + Serine + PLP \rightarrow R\text{-}S\text{-}mercaptopyruvate + PMP + H_2O$$

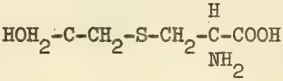
The amino acid products of reaction 1 are I and II where RSH is dithioerythritol or mercaptoethanol, respectively. The α-keto acid products of reaction 2 are III and IV when the same thiols are used. Compounds I, III, and IV have not previously been described. Their structures have been established by various synthetic and analytical techniques in this work.



$$HSCH_2\underset{H}{\overset{\overset{H}{O}}{C}}-\underset{H}{\overset{\overset{H}{O}}{C}}-CH_2-S-CH_2-\underset{NH_2}{\overset{H}{C}}-COOH$$

I

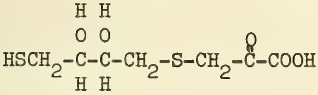
S-alanyldithioerythritol



$$HOH_2-C-CH_2-S-CH_2-\underset{NH_2}{\overset{H}{C}}-COOH$$

II

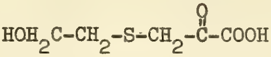
S-hydroxyethyl-L-cysteine



$$HSCH_2-\underset{H}{\overset{\overset{H}{O}}{C}}-\underset{H}{\overset{\overset{H}{O}}{C}}-CH_2-S-CH_2-\overset{O}{\overset{\|}{C}}-COOH$$

III

S-pyruvyldithioerythritol



$$HOH_2C-CH_2-S-CH_2-\overset{O}{\overset{\|}{C}}-COOH$$

IV

S-pyruvylmercaptoethanol

The study of these reactions has given useful information on the mechanism, control, and specificity of tryptophan synthetase.

(2) A new pyridoxal phosphate intermediate formed by the AB complex of tryptophan synthetase with L-serine is being investigated by spectrophotometric, optical rotatory dispersion, circular dichroism, and chemical studies. The complex is thought to involve one of the sulfhydryl groups of the enzyme. Its formation may explain the role of serine and pyridoxal phosphate in promoting aggregation of the A and B proteins and the role of the A protein in

preventing one of the reactions catalyzed by the B protein alone.

(3) The binding of pyridoxal phosphate and analogs to aspartate decarboxylase is being studied in order to determine features of the protein and cofactor necessary for binding. The binding of pyridoxal phosphate is also being studied in relation to its role in promoting subunit aggregation.

Significance to the Program of the Institute:

These studies give information on the mechanism and specificity of pyridoxal phosphate enzymes which are important in amino acid metabolism.

Proposed Course:

These studies will be continued and extended with the long range goal of identifying groups involved in substrate and cofactor binding and catalytic activity in order to understand molecular mechanisms at the active sites of pyridoxal phosphate enzymes.

Honors and Awards:

None.

Publications:

Publications other than abstracts from this project:

Miles, E. W.: α-Methylserine Transhydroxymethylase *(Pseudomonas)*. In Tabor, H., and Tabor, C. (Eds.): Methods in Enzymology. New York, New York, Academic Press. (In Press)

Miles, E. W., Hatanaka, M., and Crawford, I. P.: A New Thiol-Dependent Transamination Reaction Catalyzed by the B Protein of *Escherichia coli* Tryptophan Synthetase. Biochemistry 7. (In Press)

Serial No. NIAMD-LBP-11
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Molecular Chemistry of Enzyme Specificity

Previous Serial Number: NIAMD-LBP 11

Principal Investigator: Leonard D. Kohn

Other Investigators: William A. Warren

Cooperating Units: 1) Dr. William R. Carroll & Mr. Ellis Mitchell, Laboratory of Biophysical Chemistry, NIAMD: Ultracentrifuge studies; 2) Mr. George Poz, Arthritis and Rheumatism Branch, NIAMD: Amino acid analyses; 3) Drs. William Jakoby & Robert Allen, Laboratory of Biochemistry and Metabolism, NIAMD: Tartrate Dehydrogenase studies; 4) Dr. Roger Winand, Visiting Scientist, Laboratory of Biochemical Pharmacology, NIAMD: TSH-Exopthalmic Factor investigations.

Man Years:

Total:	2-1/2
Professional:	2
Others:	1/2

Project Description:

Objectives: The long range objective is an analysis of the molecular bases for enzyme activity and specificity as well as the mechanisms by which such factors are determined or regulated.

Methods Employed: Procedures common to the purification, assay and chemical modification of enzymes have been coupled with methods evaluating the size, shape, composition and sequence of macromolecules.

Major findings: Structure-function comparisons of "hydroxypyruvate utilizing" reductases have been initiated. Glyoxylate reductase, tartronic semialdehyde reductase, and hydroxypyruvate reductase have been found to have a subunit structure. The former enzyme, M. Wt. 100,000, is composed of 3 subunits, M. Wt. 36,000. Subunit arrangement seems to be of an AAB type, i.e. a composition of two identical and one different polypeptide chains. The native molecule has 11 total cysteine residues, each A subunit containing 5 of these. Four free sulfhydryl groups are easily titratable whereas two more are assay-

Major findings: (continued)

able on prolonged incubations. When labeled with iodoacetamide, all free -SH groups are localized in one main peptide. The enzyme is dissociated into inactive dimers and monomers; however, monomer preparations can be reassociated to form active molecules of more than one type. Reassociation is dependent on pH, temperature, buffer, and sulfhydryl reagents. Tartronic semialdehyde reductase (M.Wt. 104,000) has 4 subunits; hydroxypyruvate reductase (M.Wt. 72,000) has 3. Both have similarly low total cysteine contents.

The tartrate dehydrogenase has been purified to homogeneity and crystallized. The 155,000 M.Wt. enzyme is composed of 4 subunits (M.Wt. 38,000). Complex requirements have been found for a divalent metal cation, monovalent metal cations, and ammonium ions. The latter cause an interesting dissociation of specificity, the meso-isomer oxidation being increased while the L-enantiomorph oxidation is inhibited.

A separation of TSH and exopthalmic factor activities has been explored. Preparations have been resolved into single protein species by disc-gel analyses - with individual components retaining both activities to differing degrees.

Significance to Bio-medical Research and the Program of the Institute: Analyses of the molecular bases for activity and specificity and the mechanisms by which these determinants are influenced might conceivably offer a further understanding of the nature of metabolic and endocrine defects. This work is pursued with the hope that such understanding will aid in attacks on related disease processes.

Proposed Course of the Project: Structure-function analyses of two model systems - hydroxypyruvate reductases and the tartrate-malate dehydrogenases are being pursued. The localization of active and regulatory sites or subunits will be sought and the comparative structures of these units compared. Correlation with the kinetics of modified proteins and substrates will be attempted. Similar studies of the TSH-exopthalmic factor relationship will be initiated.

Honors and Awards: None.

Publications:

1. Kohn, L.D. and Jakoby, W.B.; Tartaric acid Metabolism III: The Formation of Glyceric Acid. J. Biol. Chem., 243:2465-71, (1968).

2. Kohn, L.D. and Jakoby, W.B.; Tartaric Acid Metabolism IV: Crystalline Malic Dehydrogenase, J. Biol. Chem., 243: 2472-78, (1968).

3. Kohn, L.D. Packman, P., Allen, R., and Jakoby, W.B.; Tartaric Acid Metabolism V: Crystalline Tartaric Acid Dehydrogenase, J. Biol. Chem., 243: 2479-85, (1968).

Publications: (continued)

4. Kohn, L.D., and Jakoby, W.B.; Tartaric Acid Metabolism VI: Crystalline Oxaloglycolate Reductive Decarboxylase, J. Biol. Chem., 243: 2486-93, (1968).

5. Kohn, L.D. and Jakoby, W.B.; Tartaric Acid Metabolism VII: Crystalline Hydroxypyruvate Reductase. J. Biol. Chem., 243: 2494-99, (1968).

6. Warren, W.A. and Kohn, L.D.; Structure-Function Relations of Hydroxypyruvate Reductases: Properties of Glyoxylate Reductase. Fed. Proc. 27: 639, (1968).

*7. Warren, W.A.; Biosynthesis of Phosphonic Acids in Tetrahymena. Biochim. Biophys. Acta, 156: 340-346, (1968).

*Work completed in this laboratory but initiated and carried forth in partial fulfillment of requirements for The Four College Cooperative Ph.D. program, Amherst College, Mass.

Serial No. NIAMD-LBP-12
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolism of Histidine

Previous Serial Number: Same

Principal Investigator: Dr. Herbert Tabor

Other Investigators: Dr Matthew Rechler

Cooperating Units: None

Man Years:

Total: 2-1/2
Professional: 1
Other: 1-1/2

Project Description:

Objectives:

Histidase from a pseudomonad has been purified and characterized. This is a requisite first step for studying the mechanism of the enzymatic reaction, and for understanding the control mechanisms involved in histidine degradation in bacteria.

Methods Employed:

Enzyme activity is determined spectrophotometrically. Purification procedures include DEAE and Sephadex chromotography, and preparative acrylamide gel electrophoresis. Physical parameters have been determined in the analytical ultracentrifuge. Amino acid analysis has been performed in an automatic amino acid analyzer.

Major Findings:

1. Homogeneous histidase preparations have been obtained. Histidase has an $s^{o}_{25,w}$ of 11.1 S, $D_{25,w}$ of $5.0 \times 10^{-7} cm^2 sec^{-1}$, and a molecular weight of 210,000. There are 20 half-cystine residues, of which 1-2 react with dithionitrobenzoate in 6M urea.

2. Guanidine hydrochloride and mercaptoethanol dissociate histidase into subunits of approximate molecular weight 35,000.

Significance:

With the purified enzyme as a tool, the reaction mechanism of this interesting non-oxidative deamination may be further explored.

Proposed Course:

Tryptic peptide mapping will allow determination of the identity of the component subunits, and enable localization of the active site of the enzyme (by affixing labeled substrate or inhibitor).

Honors and Awards:

NONE

Publications:

NONE

LABORATORY OF PHYSICAL BIOLOGY

As in former years the activities of our Laboratory span the spectrum of biomedical endeavor from the mathematical elucidation of force fields within simple inorganic molecules to aspects of integration in the human central nervous system. The availability of sophisticated apparatus and of colleagues with competence in physics and chemistry benefits the biologists with interdisciplinary problems and occasionally one of the physical scientists perceives biological implications in his methodology or material.

The Laboratory continues to exercise a gratifying attraction for postdoctoral visitors from abroad and we have benefited this year from association with colleagues from Australia, Canada, England, France, Italy, Japan and The Netherlands.

In the summaries below only the names of the principal LPB investigators involved in a specific study are given. The names of technical assistants, investigators from other NIH units and extramural collaborators will be found in the individual reports.

Molecular Forces and Structure

Even for simple phosphorus and arsenic halides of unequivocal composition and known bipyramidal structure the infrared spectra have a complex fine structure of multiple narrow absorption bands corresponding to various rotational, vibrational, bending, and combined interatomic forces. Last year it was shown that the parameter that combines rotational and vibrational forces (the Coriolis or zeta function) can be adequately determined from "band contour" measurements in which several fine lines are lumped. By mathematical analysis these Coriolis functions can then be used to distinguish the absorption bands corresponding to axial and in-plane bending. The Coriolis constant has also been used, in combination with the band shifts that occur with isotope admixture, to identify specific force fields in SiF_4 in the gas phase at low temperature. Our new helium-neon laser spectrometer has been used to obtain Raman spectra of C_6Cl_6, C_6Br_6, C_6I_6 and C_6F_6 showing that whereas in the last-named molecule the halogen atoms are in the plane of the benzene ring they are staggered in the other three molecules so as to produce a "corrugated" configuration (Levin).

The origin of absorption bands in the ultraviolet spectra of simple organic molecules has been investigated by quantum mechanical computation of electronic energy levels. By partial substitution of heavy hydrogen one of the transitions of ethylene molecule has been identified as torsional. In methylated ethylenes the cause of a transition band at 200 mμ has been identified and several of the energy levels of the methyl radical have been calculated, the value for the lowest transition being unexpectedly high (McDiarmid).

Nuclear magnetic double resonance spectroscopy of aniline, methyl amine and quinoline with substituted heavy nitrogen has shown that hydrogen bonding has a pronounced effect on the nitrogen resonance when the N acts as a proton acceptor but has only a small effect when it acts as the donor. NMDR has also permitted the determination of the relative signs of spin-spin coupling constants (Paolillo, Becker). NDMR appears promising for elucidating the structure of certain pigment molecules of the Elsinochrome type (Paolillo and Lousberg). In certain substituted pyrimidines Shoup and Becker found a marked solvent dependence for the rate of internal rotation of the $N(CH_3)_2$ group in 1,7,7-trimethylcytosine. The new laser spectrometer has permitted some refinement of previous measurements of molecular vibrational modes in p-benzoquinone (Saum, Becker).

A study of the behavior of the diketone camphorquinone in polarized ultraviolet light and in the infrared indicates that the two ketone groups undergo extensive coupling (Charney). A similar study of crystalline benzil is underway (Charney, Eaton). A new electro-optical effect in which absorption of polarized light in two axes by molecules exposed to strong electric fields, applied to DNA, indicates an unexpectedly linear (rather than quadratic) dependence of the dichroism on field strength, and possibly identifies a hitherto unobservable transition (Charney, Yamaoka). In the synthetic polypeptide poly-γ-benzyl-glutamate, electro-dichroic measurements indicate a phenyl-peptide carbon optical interaction extending across 6 bonds, 3 of which should be reasonably free to rotate (Charney, Milstien).

Spectrochemical methods applied to extracts of the pigmented mold Elsinoe have completed the structural elucidation of the three red pigments. Related pigments from two aphids are under investigation (Weiss). The structure of a codeine derivative has been elucidated (Eisner, Weiss) and the structure and mode of formation of several photodimers were clarified (Ziffer, Williams). Edwards and Weiss worked out a simplified synthesis of pentalanes and propellanes.

The order of toxicity of a series of acridine compounds to gram-positive bacteria seem to correlate well with calculated electron density at the ring nitrogen and the energy of the lowest empty molecular orbital (Sharpless).

The time courses of the complexing of two purified phosphatides from beef retina with the rhodopsin chromophore retinal have been followed spectroscopically (Adams).

Macromolecular Structure and Behavior

Spectroscopic and optical rotatory dispersion measurements of complexes between the dye acridine orange and various DNA derivatives indicate that AO protects DNA against thermal denaturation, that heat and acid-denatured DNA still contains some ordered structure and that the Cotton effect is not a reliable index of order in tertiary configuration of macromolecules (Resnik, Yamaoka).

Electron spin resonance measurements of NO-hemoglobin show that dehydration alters the structure around the heme group in a manner similar to detergent and that sodium salicylate, at a concentration known to enhance oxygen uptake of oxyhemoglobin, changes the absorption to a Fe^{+++} type (Kon).

ESR spectra of Co (II) perchlorate derivatives identified a hitherto unknown and truly hexacoordinated phenylisocyanide compound (Kataoka).

Optical rotatory dispersion and circular dichroism applied to actinomycin D and its complex with DNA elucidated the relations between the lactone rings and optically active bands and, applied to unsaturated ketones, identified certain transitions (Ziffer, Yamaoka).

Optical properties have helped specify the arrangement of the five molecules of bacteriochlorophyll within pigment-protein supermolecules, and spectroscopic study of fluorescent complexes between bacteriochlorophyll and the dye rhodamine have suggested how and where energy is transferred between dye and photopigment (Olson, Jennings).

The activation energies for aggregation of sickle-cell hemoglobin into microtubules and for aggregation of galactothermin molecules both prove to be about 25 kilocalories per mole. In human blood platelets microtubules appear to be involved in pseudopod formation (Murayama).

Further data from electron microscopy and x-ray diffraction on bovine-liver catalase crystals suggest that the molecule, rather than being strictly spherical, has four nearly equal subunits arranged at the corners of a tetrahedron. If these subunits are spherical in shape they will have a diameter of about 50 Å. A new collagen related fibrous structure has been observed in the electron microscope as 200 Å diameter spheres spaced 600 to 700 Å along a thin fiber which itself can be shown to be a bundle of smaller fibers, suggesting tropocollagen. These strands are unaffected by collagenase (Labaw).

Membrane and Transfer Phenomena

It has been shown that two steroid hormones, in 10^{-7}M concentration, can increase the rate of polymerization in alkyl phosphate films by 30-40 fold (Gershfeld). The first direct evidence has been obtained that hydrocarbon chains in an ordered 2-dimensional array can contribute a considerable stabilization energy to structures in which the hydrocarbons are present. The energy of cohesion amounts to about -23 K cal/mole for an 18 carbon chain (Gershfeld, Muramatsu).

Several liquid membranes composed of quaternary ammonium compounds in organic solvents have been found to yield concentration potentials for monovalent halides almost equal to the theoretical maxima. The potentials were found to be independent of dielectric constant of solvent over at least a 10-fold range, and of concentration of active compound in the membrane phase over a 1000-fold range (Sollner, Shean).

Measurements using cation, specific glass electrodes and isotopically labelled ions showed that, contrary to the generally accepted view, sodium ion does not bind tenaciously to polyacrylate polymer but rather that both electrical transfer of sodium ion and the exchange with polymer-associated ion follow the theoretical rates. Anomalous results in the literature are due to excessive and previously undetected electro-osmosis (Gottlieb).

Direct microelectrode recording of fast electrical transients from the retina upon local illumination show that current flow is proportional to rate of decay of lumi-rhodopsin, an intermediate in the photolysis of rhodopsin, and indicate that rhodopsin is in the membrane of the distal rod segment, probably in the microvilli (Hagins, McGaughy). Freeze-dried retinal sections, investigated via the Bureau of Standards electron microprobe, have yielded promising indications of concentration differences of several inorganic ions between tissue layers (Tormey).

The giant photogenic cells of the luminous phengodes larva are strongly fluorescent and have a cortical structure with profuse microvilli (Bassot).

The widely used technique of precipitating enzymatically liberated phosphate with lead was tested in muscle cells (where the location of myofibrillar ATPase sites is known) by activating both myofibrillar and reticular ATPase sites with calcium applied in the presence of lead. Electron dense deposits were found to be associated with the internal membranes but not with the myofibrils, even though the myofibrils had undergone active contraction. This observation indicates that deposit localization in the presence of lead is neither necessary nor sufficient evidence for localized phosphatase activity in biological tissues (Podolsky).

Contraction in calcium-loaded skinned fiber segments was found to be accompanied by substantial release of calcium from the sarcoplasmic reticulum (Ford). Since contraction can be coordinated along the entire length of the fiber segment used in these experiments (~ 1 mm), calcium release is probably triggered by a regenerative process involving the internal membrane system.

A microdiffusion technique was developed for measuring the mobility of ions and molecules within single muscle cells (Kushmerick). Calcium diffuses 10-100 times more slowly than it does in water, showing that it is sequestered by the internal membrane system. ATP appears to be freely diffusible within the cell.

In hyperosmotic solution the action potential of a muscle fiber is normal but the contraction rate is reduced. This change is due to a decrease in myofibrillar contractility rather than interference with activation, and it is produced by the increase in intracellular ionic strength associated with loss of water from the cell (Sugi).

Additional work on Pteroptyx fireflies from Thailand has confirmed last year's remarkable finding (in Luciola) that the fine nerves in photogenic organ end in contact with the membranes of all of three diverse cell types. Since it is known that luminescence is neurally excited the junctional mechanism must be very different from those in conventional neuroeffectors (Peterson).

Physiological and Metabolic Processes

Hydras regenerated apical ends within 48 hours of "decapitation" and during the whole period the frequency of mitotic divisions was reduced (Park, Blankenbaker). In unfertilized eggs of a green hydra symbiotic algae were already present, and in an hermaphroditic species no self-fertilization occurred (Ortmeyer).

An inhibitor for light production by Photobacterium fischeri, present in standard nutrient broth, was shown to bind directly to luciferase molecules though not affecting luciferase synthesis (Kempner, Hanson). All enzymes in cells of Euglena stratified by centrifugation were found to be bound to particulates (Kempner).

Experiments on rats stressed by acute exercise or by hypoxia (simulated altitude) showed alterations in key metabolic enzymes in lymphatic tissues leading to increased glycolysis at the expense of direct hexose monophosphate shunt metabolism of glucose (Dieter). Certain serum aldolases, transaminases and dehydrogenases were also elevated by simulated altitude and dimethyl sulfoxide administration although previous exercise at normal atmosphere pressure reduced or prevented most of these effects (Altland). Effects of hypoxia were least if produced by $He-O_2$ mixtures than if produced by volumetrically equivalent N_2-O_2, $A-O_2$ or SF_6-O_2 mixtures. These effects were due to the effect of helium in reducing body temperature and improving arterial oxygen concentration.

During the larval development of a blowfly eight dipeptides and one tripeptide have been identified, all with either histidine or lysine C terminal. Such peptides have not hitherto been found free in nature (Levenbook). The aldolases from larval and adult fat body are different (isozymes) although the adult fly muscle aldolase is apparently identical with mammalian aldolase (Bauer).

Synchronously flashing pairs of Thai fireflies, recorded with separate photometers, exchange the lead frequently and may maintain a phase difference averaging only 8 msec in a flashing period of 600 ±7 (s.d.) msec (J. Buck, Bassot). Human subjects tapping telegraph keys in synchrony with an external sonic signal use anticipatory cueing from both their own preceding tap periods and from preceding tap-signal phase differences (J. Buck, E. Buck, Brubach).

Serial No. NIAMD-LPB-1
1. Physical Biology
2. Cellular Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Mechanism of Muscular Contraction

Previous Serial Number: Same

Principal Investigator: R. J. Podolsky

Other Investigators: H. Sugi, L. E. Ford, M. J. Kushmerick

Man Years

Total:	5 1/4
Professional:	3 1/4
Other:	2

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion of contracting muscle fibers.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of "skinned" muscle fibers under various conditions.
4. Measurement of mobility of radioactively labeled substances in single cells.

Major Findings:

1. The internal membranes of skinned muscle fibers are capable of releasing calcium ions into the myofibril region of the cell by a regenerative mechanism, suggesting that such a process is a step in normal activation.
2. Muscle cells have a normal action potential in a Ringer solution with elevated osmotic pressure, but the contractile response is eliminated. This is due to the inhibitory effect of the increased intracellular ionic strength (resulting from the loss of water from the cell) on the contractility of the myofilaments.

3. Phosphate released from ATP by the myofilament reactions which drive contraction is not precipitated locally by lead ions, even though electron dense deposits are associated with the adjacent intracellular membranes. This negative result shows that local precipitation by lead does not necessarily accompany phosphate production by tissue enzymes.
4. Calcium ions diffuse 10-100 times more slowly in muscle cells than in water.

Significance to NIAMD Research:

The elucidation of the molecular mechanism of muscular contraction, together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. It is generally believed that the contraction-relaxation cycle in muscle cells is due to movements of calcium ions between the internal membranes and the myofibrils. The actual calcium movements will be measured in skinned muscle fibers to see whether the kinetics are consistent with the behavior of intact cells.
2. The influence of various agents (deoxycholate, metal ions, ionic strength) on the relation between pCa and contractile force will be examined in skinned muscle fibers to get additional chemical information about the force generating process.
3. Physical properties of myofilament cross links will be deduced from the instantaneous elasticity of contracting fibers. Such experiments are interpreted by computer simulation of contractile models.
4. Interaction of ATP, Na, K, and Ca ions with intracellular structures will be studied in isolated muscle cells by microdiffusion techniques.

Publications:

Tice, Lois W.: _In situ_ studies of the sarcotubular system. In Tang, Ralph D., Kavaler, Fred, and Roberts, Jay (Eds.): _Factors Influencing Myocardial Contractility_. New York, Academic Press, Inc., 1967, pp. 339.350.

Podolsky, R. J.: Deposit formation in muscle fibers following contraction in the presence of lead. _J. Cell Biol_. In press.

Podolsky, R. J.: Membrane systems in muscle cells. In _Aspects of Cell Motility_, Cambridge University Press. In press.

REPORTED IN PRESS - 1967

Podolsky, R. J.: Recent studies of the activation and contraction of striated muscle fibers. In Milhorat, A. T. (Ed.): *Exploratory Concepts in Muscular Dystrophy and Related Disorders*. Amsterdam, Excerpta Medica, 1967, pp. 277-278.

Serial No. NIAMD-LPB-2
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Triggering of Bioluminescence. Sense of Rhythm.

Previous Serial Number: Same

Principal Investigator: John B. Buck

Other Investigators: J. M. Bassot, Howard F. Brubach and Miriam R. McLean

Cooperating Units: Mrs. Elisabeth M. Buck, Guest Worker

Man Years:

Total:	5
Professional:	2
Other:	3

Project Description:

Objectives:

Neuro-effector coupling in bioluminescence; central nervous pacemaking, sequence judgement and phase-shifting.

Methods Employed:

Standard electrophysiological; multichannel chart recording with computer statistical inventory.

Major Findings:

1. Mrs. McLean has completed an extensive manuscript, in collaboration with Mrs. Lesley Ballantyne of the University of Queensland, on the taxonomy of Pteroptyx fireflies of Southeast Asia. This work, beside being a valuable scholarly contribution in its own right, has validated the species names needed for use in a definitive publication on synchronous flashing referred to below. Mrs. McLean is also assembling for publication and supplementing several years accumulation of data on firefly development and culture. This paper should help fill the often reiterated need for a laboratory culture method for fireflies which can supply material through the year.

2. Thanks to material brought from Thailand by Dr. Robert Gordon (CI-NIAMD) the analysis of synchronous flashing in Asiatic fireflies and its comparison with rhythmic and synchronous activity in human beings was completed and published (Buck and Buck, 1968). Measurements of the neural latency to electrical stimulation, made with the help of Dr. Frank Hanson, formerly in our laboratory, provided the crucial evidence that the synchronous flashing involves an anticipatory or predictive mechanism dependent on ability to measure time from and discriminate temporal sequence in, previous activity cycles. It is an interesting commentary on the fruitfulness of the comparative physiological approach that the firefly synchrony, originally studied for its own sake, led to new findings about human timekeeping (see below) which were then in turn, applied to further elucidate the firefly phenomenon. In the overall outcome both firefly synchrony (involving oscillators which are nearly fixed-frequency and with little central nervous lability) and human synchrony with much lability but much susceptibility to extraneous influence) were needed for an understanding of the underlying mechanisms.

3. Visiting Scientist J. M. Bassot has engaged in extensive histological and electron microscopical studies of local fireflies and of the remarkable larviform female of the beetle Phengodes (courtesy of Dr. Albert Carlson of the State University of New York) which is especially interesting for its possession of gigantic isolated photocytes.

4. In collaboration with H. F. Brubach and E. Buck the study of human synchronization and rhythm was pushed hard and preliminary results presented at the AAAS convention. Our evidence indicates that human subjects can differ markedly in ability to maintain a steady endogenous motor rhythm, and, independently, in ability to synchronize. In fact what is ordinarily called "sense of rhythm" is really "sense of synchrony" and some people have good rhythm combined with relatively poor synchrony. In an attempt to localize more closely the neural mechanisms of these abilities we have been concentrating on identifying the cue or cues, which, feeding back via proprioceptive and auditory channels, make possible sequence discrimination. We have also worked at explaining the curious sort of temporal bias shown by most individuals, in which the subject tries to act in exact synchrony with a rhythmic signal and thinks that he is succeeding but in fact may actually lead the signal by a substantial amount (e.g. 50 msec).

Significance to NIAMD Research:

It is hoped that our present strong interest in the comparative physiology of rhythm and synchronization will eventually elucidate the hitherto almost entirely neglected question of how human beings (alone of all warm blooded animals) are able to synchronize motor acts to external rhythms. Such information on normal human physiology will not only provide a rational basis for measuring understanding synchronous human activities such as marching, dancing and musical performance, but for comparing quantitatively the abilities of different individuals and quite possibly for diagnosing analytically deficiencies in such ability due to accident or disease.

Proposed Course of Project:

We propose to continue to push the study of synchrorhythms in both invertebrates and vertebrates. We intend specifically to look for electrical CNS activity ("brain waves") associated with voluntary rhythm and synchronic activity and to compare normal performances in patients with impaired sensory and motor function.

Honors and Awards:

Invited by the University of California to lead an International Bioluminescence Expedition.

Publications:

Buck, John and Buck, Elisabeth: Mechanism of rhythmic synchronous flashing of fireflies. Science 159: 1319-1327, March 1968.

Serial No. NIAMD-LPB-3
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Proteins, Peptides and Free Amino Acids in the Blowfly, *Phormia regina*

Previous Serial Number: NIAMD-LPB-3

Principal Investigator: L. Levenbook

Other Investigators: A. Bauer

Cooperating Units: Dr. R. Bodnaryk, Visiting Fellow

Man Years:

Total: 2
Professional: 2
Other: 0

Project Description:

Objectives:

1. To determine the amino acid sequence of several naturally occurring larval peptides of the blowfly.

2. To identify and characterize enzymes concerned with the synthesis and breakdown of the above peptides during larval growth.

3. To compare the free amino acid pattern with the amino acid composition of the bulk protein of the tobacco hornworm during metamorphosis.

4. To further characterize *Phormia* aldolases.

Methods Employed:

A wide variety of biological and biochemical techniques.

Major Findings:

1. Employing standard procedures of peptide chemistry, eight dipeptides and one tripeptide have been unequivocally identified. The common pattern emerging was that histidine or lysine was always C-terminal. Such peptides have not hitherto been found in nature. Earlier experi-

ments on the isolation and sequencing of a male sex-peptide known to occur in other Diptera were unsuccessful due to lack of a suitable assay.

2. Interest has arisen in the composition of the tobacco hornworm (Protoparee sexta) meconium. At the time of voiding, the meconium is very rich in uric acid, a major basic component which we have now characterized (and estimated) as free histidine, and many other identified free amino acids. To ascertain the source of these amino acids, and particularly of the histidine, it was decided to follow the free amino acid and protein amino acid profiles during metamorphosis. The first few determinations however showed that the speeded-up Piez-Morris amino acid analysis procedure, as published, was ineffective in resolving ammonia, lysine and histidine when these were concurrently present in widely disparate amounts, as in this insect. Accordingly, over 150 runs extending over several months, have been expended in trying to devise a rapid, single column procedure with adequate resolution for these basic amino acids. It is concluded that this objective is simply unattainable with the best resins currently available, and the amino acid analyzer is presently being modified to the rapid, two-column system which will resolve the amino acids.

3. To "terminate the completion" of our aldolase studies, the molecular weights of the larval and adult enzymes have been determined and found to be identical (viz. 160,000) with mammalian aldolase. Further, it has been shown that the adult fly aldolase, again like the mammalian enzyme, has three C-terminal tyrosines; and that if these are removed the residual enzyme loses most of its activity towards fructose diphosphate but not towards fructose-1-phosphate. It has also been found that aldolase from larval and adult fat body are electrophoretically different (i.e. they are isozymes).

Significance to NIAMD Research:

During insect metamorphosis profound cellular dedifferentiation and subsequent differentiation occur in one and the same organism. This biological material is therefore particularly favorable for a study of protein interconversion, and changes in free amino acids and of peptides during protein breakdown and synthesis.

Proposed Course of Project:

1. To study the mode of synthesis and breakdown of the Phormia peptides, and hopefully, to prepare from the larvae a purified enzyme that will permit us to synthesize doubly-labeled peptides for subsequent injection, this being the method of choice to study their ultimate fate.

2. Continuation of analyses of tobacco hornworm protein and free amino acids during metamorphosis.

3. Study of the composition of the hornworm meconium.

4. To purify aldolases from several grams of isolated *Phormia* fat body and muscle, in sufficient amounts to make meaningful chemical comparisons.

Serial No. NIAMD-LPB-4

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Molecular Structure and Behavior of Hemoglobin.

Previous Serial Number: NIAMD-LPB-4

Principal Investigator: Makio Murayama

Other Investigators: None

Cooperating Units: Dr. Arthur L. Schade, NIAID-LID-63-A

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

1. To find a molecular mechanism of the "unsickling" of erythrocytes from sickle cell anemia patients; investigate the thermodynamic interrelationship between the molecular mechanisms of "unsickling" under high hydrostatic pressure and "unsickling" by cooling to the ice point.

2. To study galactothermin, a new protein faction in human milk which aggregates upon warming to 38° but deaggregates upon cooling in an ice bath (to water clear solution). The protein has been designated "hydrophobic fraction-x" now called galactothermin. Because galactothermin and S hemoglobin both have a negative temperature coefficient of aggregation, the influence of high hydrostatic pressure upon the rate of aggregation is investigated.

3. To find out the influence of high hydrostatic pressure on microtubules of sickled erythrocytes as well as those in human blood platelets.

Methods Employed:

In addition to those previously described, an optical cell which permits *in situ* observations of protein solution under high hydrostatic pressure is in use.

Major Findings:

The scale model of human hemoglobin molecule was rebuilt to the scale of

1/2" to Angstrom (magnified 127 million times) according to Perutz's most recent data.

The enthalpy change (ΔH) of "microtubules" formation from purified solution of human S hemoglobin is approximately 23 kilocalories mole^{-1}.

The enthalpy change (ΔH) for galactothermin to aggregate is about 26 kilocalories mole^{-1}.

Human blood platelets at 38° and 1 atmosphere form pseudopods which appears to be bundle of microtubules.

As suspected, blood platelets are essentially spherical under 10,000 psi (about 600 atmospheres); microtubules do vanish.

Significance to NIAMD Research:

As previously stated, sickle cell anemia is a hereditary hemolytic anemia; it is one of the best studied inborn-errors of metabolism; it is a metabolic disease. For these and other reasons the investigation of this metabolic disease is quite significant for the metabolic disease institute.

Blood platelets aggregate to form thromboses. Microtubules in blood platelets as well as those in sickle cells are thermodynamically similar. Investigations of this type could throw some light on the energetics of self-assembly of cell organelle.

Proposed Course of Project:

The molecular mechanism of unsickling of sickled erythrocytes will be explored further from the standpoint of thermodynamics.

Similarities and differences in the process of microtubule formation in sickled erythrocyte and blood platelets will be explored.

Publications:

Murayama, M.: Magnetic orientation of sickled erythrocytes. In Sunderman, F. W., and Sunderman, F. W., Jr. (Eds.): The Clinical Pathology of Infancy. Springfield, Ill., Charles C. Thomas Co., 1967, pp. 16-18.

Murayama, M.: Bilirubin metabolism in the liver. In Sunderman, F. W. and Sunderman, F. W., Jr. (Eds.): Diagnosis of Liver Diseases. Philadelphia, Pa., J. B. Lippincott Co. In press.

REPORTED IN PRESS - 1967

Murayama, M.: Structure of sickle cell hemoglobin and molecular mechanism of the sickling phenomenon. *Clinical Chemistry* 14: 518-588, July 1967.

Honors and Awards:

Participated in Federation of American Societies for Experimental Biology closed-circuit TV program (Channel 2), Atlantic City, New Jersey, on Tuesday, April 16, 1968. The title of the program was "Inborn Errors of Metabolism".

Serial No. NIAMD-LPB- 5
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Hydroid Physiology

Previous Serial Number: NIAMD-LPB-5

Principal Investigator: Helen D. Park

Other Investigators: Anne B. Ortmeyer
Dwight P. Blankenbaker

Cooperating Units: Mr. Robert J. Toni,
Montgomery Junior College
and Flow Laboratories

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

1. To study the basic mechanisms (cell division, cell differentiation, and cell movement) in regeneration of apical structures in Hydra pseudoligactis.

2. To study antigenic differences between two species of Hydra.

3. To study the formation and hatching of eggs in four species of Hydra.

4. To study the effect of temperature on growth and asexual reproduction in Hydra littoralis.

5. To study morphogenesis in micro-cultures of small explants and/or dissociated cells of Hydra.

Methods Employed:

Those previously reported, and in addition the following:

1. Mr. Toni at Flow Laboratories used three serological methods in testing for antigenic differences between two species of Hydra.

2. We are still devising methods for dissociating hydra cells, and separating them from unwanted nematocysts by centrifugation; we are also devising methods for freeing whole animals and cells of contaminants; lastly, we are working out methods for growing the the cells in micro-culture using both hanging drop and Sykes-Moore chamber preparations.

Major Findings:

1. During Mr. Blankenbaker's stay as a summer employee, we completed the counting of mitotic figures in histological preparations of regenerating hydras. The results of the regeneration study were as follows:

   a. Reconstitution of apical structures was completed in 48 hours.
   b. Mitotic activity in each of the four major types of cells was depressed below that in the uncut controls, during the entire period of regeneration. Only when regeneration was complete (48 hours) was mitotic activity equal to that in the controls.

   The finding that a hydra can replace apical structures without an increase in cell division is not surprising in view of the 1958 finding of Dr. Park that a hydra can regenerate apical structures following a dose of 30,000 r of X-rays. A hydra apparently, largely makes a new head by changing the geographical relationships of its cells to each other, and by differentiation of cells present at the time of decapitation (morphollactic regeneration).

   The results of the regeneration study were presented by Dr. Park before the Developmental Biology Section of the American Society of Zoologists, December, 1967.

2. The results of the study with Mr. Toni on antigenic differences between two species of hydra are extremely preliminary and will not be presented formally until further experiments are done. We were not able to show antigenic differences using three standard serological tests, but we did learn that such a study is practicable if larger quantities of antigen, and more sensitive tests are used. So far as we are aware, no study of antigenic differences among species of _Hydra_ has been reported.

3. The study with Mrs. Ortmeyer on the formation and hatching of eggs in four species of _Hydra_ is complete and the results are in rough-draft manuscript form. The most important finding (on the basis of observing 197 eggs) was the extreme variability in hatching time -- 17-196 days -- within a species. Other observations, such as the presence of algae in unfertilized eggs in _Chlorohydra viridissima_, the developmental stage at which the hydra hatches, and absence of self fertilization in hermaphroditic species, confirm those of

earlier workers.

4. A finding from the temperature study, additional to those reported last year, is that the growth and differentiation of a bud has a very much larger temperature coefficient than does the initiation of a bud. This finding may some day have relevance in the elucidation of the difference in mechanisms involved in the two processes. The finding is of interest now, in view of the old (1958) observation of Park and the more recent (1967) one of Clarkson and Wolpert that high energy radiation has little effect on the growth of a bud once it is initiated, but a profound effect on bud initiation.

5. The study of morphogenesis (cell division, cell, differentiation, cell movement) in micro-culture is still in a most preliminary stage. We have directed our principal efforts thus far toward solving two very basic problems:

   a. Developing a salt solution of composition and concentration suitable for small explants or dissociated cells of Hydra. More than 20 different salt solutions have been tested on whole animals, then on hanging drop or Sykes-Moore chamber preparations. In one of these solutions two types of cells were alive after 132 hours at 15° C (optimum temperature for growth of whole animals).
   b. Freeing whole animals and/or cells of contaminants. Some success has been achieved against extra-cellular contaminants by using a mixture of antibiotics. However, intra-cellular contaminants have persisted.

   Hydra cell culture has been attempted by other workers (Burnett, Li, Fulton, Tardent and Sklaer) but without success.

Significance to NIAMD Research:

This work can be considered as contributing to the basic biology of the mechanisms of cell division and cell differentiation.

Proposed Course of Project:

Using techniques of hanging drop and Sykes-Moore chamber culture we plan to study cell division, cell movement and cell differentiation of dissociated cells and small, tissue explants.

Serial No. NIAMD-LPB-6
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Ultrastructure of Firefly Light Organs

Previous Serial Number: NIAMD-LPB-6

Principal Investigator: Margaret K. Peterson

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

A study of the fine structure of the light organs of a number of species of adult fireflies, and the correlation of anatomical differences with observed behavioral and physiological differences, with the ultimate objective of elucidating the neural control mechanisms of light production.

Methods Employed: Standard Electron Microscopy.

Major Findings:

A detailed study of the light organs of four species of fireflies from southeast Asia and of a number of American species revealed that, although the general structure of the light organs is similar in all species studied, there are several significant differences in detail. In the Asian species, nerve endings make direct contact with the photocytes, as well as with tracheolar cells, while in American species thus far studied they are separated from the photocytes and make contact only with the cells of the tracheal end organ. In the Asian species, tracheoles are more intimately associated with the cytoplasm and organelles of the photocytes, penetrating into the interior of the cells, whereas in American species they are found only between the cell membranes. In the Asian species, the photocyte granules are larger and more elongated than in American species, and they contain more numerous microtubules. A paper describing these findings and attempting to interpret their significance is nearly ready for publication.

Significance to NIAMD Research:

This study should be a contribution to our knowledge of biological neuroeffector structures and mechanisms in general and of the neural control mechanisms of firefly flashing in particular.

Proposed Course of Project:

We propose to study next the fine structure of the light organs of larval fireflies. The adult fireflies studied emit their light in discrete flashes, while the larvae emit their light in a steady glow. We know that the tracheal end organs characteristic of flashing adult lanterns are absent from larval organs, but the type of innervation and other details of structure remain to be studied.

Serial No. NIAMD-LPB-7
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Optical Rotatory Dispersion of the Reaction of Acridine Dyes with Macromolecules

Previous Serial Number: NIAMD-LPB-7

Principal Investigator: Robert A. Resnik

Other Investigators: None

Cooperating Units: Dr. Kiwamu Yamaoka, NIAMD-LPB-

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: A continuing study of the reaction of acridine compounds with nucleic acids.

Methods Employed: Optical rotatory dispersion and absorption spectra.

Major Findings:

1. Changes in the extrinsic Cotton effects and absorption spectra of acid and heat denatured DNA indicate, when compared to the results for native DNA, that acridine orange (AO) protects DNA against thermal denaturation at room temperature.

2. The ORD curves and absorption spectra recorded for the AO complex of formylated DNA indicate that heat and acid-denatured DNA still contain some ordered structure, probably due to base-base interactions. This conclusion is based on the relationships between the extrinsic Cotton effects of AO complexes of apurinic acid and formylated DNA compared to those for denatured and native DNA.

3. Extrinsic Cotton effects were observed with complexes of AO and native DNA under conditions where an excess number of dye molecules were present. The absorption spectra were relatively insensitive indicators of the changes that occurred in the system.

4. The ORD curves of AO complexes of apurinic acid were formerly thought to be invariant as a function of P/D. This is not so; two types of Cotton effects have been observed. The absorption spectra, however, all appear to belong to a single family of curves.

5. Changes in ionic strength as well as the salt used influence the intensity of the extrinsic Cotton effect observed with AO complexes of APA.

6. No extrinsic Cotton effect was detected when proflavine was added to apurinic acid.

7. Induced optical activity was observed when AO was added to polyglutamic acid under conditions where polyglutamic is in the random coil conformation.

The findings listed here, particularly 1, 2, 6, and 7, indicate the need for reevaluating the relationship between the structure of polymers and the occurrence of induced optical activity.

Significance to NIAMD Research:

As a result of *in vitro* and *in vivo* studies the acridine dyes are known to produce certain biochemical and genetic alterations in biological systems. A molecular basis for these effects stems from the fact that the acridines interact with polynucleotides, RNA, and DNA. The knowledge of the nature of these interactions is still less than complete.

Proposed Course of Project:

None, because of the departure of the principal investigator.

Serial No. NIAMD-LPB-8
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Structural basis of fluid transport across epithelia

Previous Serial Number: NIAMD-LPB-8

Principal Investigator: John McD. Tormey

Other Investigators: None

Cooperating Units: Dr. Jared Grantham, Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute
Dr. William Hagins, Laboratory of Physical Biology, NIAMD
Dr. Kurt Heinrich, Analytical Chemistry Division, National Bureau of Standards

Man Years

Total: 1
Professional: 1
Other: 0

Project Description:

Objective:

To identify the specific subcellular structures involved in fluid transport across epithelia and to elucidate their functional roles. Special attention is being directed toward developing techniques for localizing transport functions.

Methods: Light and electron microscopy; osmometry; electron microprobe analysis.

Major Findings:

(1) An attempt has been made to trace the route of water movement across the wall of the gall bladder by using the protein horseradish peroxidase as a histological marker. If water is transported across the gall bladder epithelium via intercellular spaces, the tracer should be excluded from the spaces by the bulk flow of water out of them and admitted to the spaces in the absence of transport. Existing methodology with this tracer has been found inadequate: the protein is able to

diffuse through the tissue for several minutes after the initiation of fixation. A technique for instantly immobilizing the tracer has therefore been devised, using quick freezing, frozen sectioning and freeze substitution. Preliminary results appear to verify the path of fluid transport via the intercellular spaces.

(2) An electron microscopic study of isolated renal proximal convoluted tubules has been carried out in collaboration with Dr. Grantham. Using the physiological techniques developed in his laboratory, single tubules dissected from rabbits were subjected to three types of in vitro condition: (1) No net transport, (2) Active isotonic salt transport, (3) Passive fluid movement in response to an osmotic gradient. No significant structural differences between conditions (1) and (2) have been found. State (3) results in massive cytoplasmic vacuolization. The relationship of this vacuolization to fluid movement is currently under study.

(3) The mechanism of fluid transport across the gall bladder wall apparently entails the presence of sizeable osmotic gradients in the region of the epithelium. An effort to localize these gradients has been made by examining frozen sections in a special microcryostat under high dry light microscope objectives. It was hoped that as the temperature of the tissue was slowly raised, regions of local melting would be observed. Heretofore unappreciated limitations to the spatial resolution of this technique were found instead. Even under ideal conditions the best resolution is on the order of hundreds of microns. It will therefore be reported in the literature that this method appears useless for the subcellular localization of osmotic gradients.

(4) The quantitative localization of electrolytes within tissues is an essential step in the investigation of both electrolyte transport systems and many other physiological problems. Electron microprobe x-ray analysis has spectacular potentialities for identifying inorganic ions in situ. However, until now, there has been a lack of satisfactory techniques for preparing tissue samples for the probe in such a way that translocation of diffusible materials could not occur. In collaboration with Dr. Hagins an apparently satisfactory technique for preparing specimens of retina has been devised; the method involves quick freezing of isolated retinas, frozen sectioning at -35°C and freeze drying. Microprobe analysis of these specimens by Dr. Kurt Heinrich has demonstrated apparently significant variations in concentrations of Na, K, and Cl among the layers of the retina. Efforts are now being made to establish the quantitative reliability of the localizations observed.

Proposed Course of the Project:

The project is being terminated as of June 30, due to the transfer of the Principal Investigator from NIH to the University of California at Los Angeles. It is hoped it will be continued there with the aid of extramural support from NIAMD.

Significance to NIAMD:

Combined anatomical and physiological approaches, such as employed in this project, seem basic to the development of better understanding of the mechanism of fluid transport across epithelial membranes. Such understanding is essential for the progress of knowledge of salt and water metabolism throughout the body in health and disease.

Honors and Awards: Invited panelist, Symposium on Cystic Fibrosis, N.Y.C., February, 1968. Chairman, Symposium on Electron Microscopic Approaches to Biological Transport, Washington Society for Electron Microscopy, May, 1968.

Publications:

Tormey, John McD., and Diamond, Jared M., The ultrastructural route of fluid transport in rabbit gall bladder, J. Gen. Physiol., 50:2031-2060 (Sept.) 1967.

Serial No. NIAMD-LPB-9
1. Physical Biology
2. Electrochemistry and
   Colloid Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The interaction between small ions and polyelectrolytes in solution

Previous Serial Number: None

Principal Investigator: Melvin H. Gottlieb

Other Investigators: None

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

To determine the effects of the strong electric fields which exist in the neighborhood of some charged biopolymers on the kinetic mobility of small ions in their vicinity.

Methods Employed:

Since this is the first report on this project, some background will be outlined. The binding of small ions, such as Na^+, K^+, or Cl^-, to biopolymer molecules with few exceptions has only been studied by methods which give the thermodynamic activity coefficients of these ions. These methods can not indicate whether the ions are "bound," in the sense of having lost their kinetic freedom. A number of years ago F. T. Wall and his coworkers reported on electrical transference measurements which indicated that in solutions of sodium polyacrylate, a large fraction of the Na^+ ions moved with the polyacrylate ions towards the anode, and hence were apparently "bound" to these ions ⌜J.A.C.S. 72: 2636 (1950)⌟. Further, from measurements made on the unidirectional rates of movement of Na^+ ions in similar electrical transference experiments, it was concluded that the rates of exchange between free and bound Na^+ ions were extraordinarily slow, characterized by half-times up to 10 minutes (J. Chem. Phys. 20: 1200-1207, 1952).

The original plan of this project was to determine the rate of exchange between free and bound ions in certain bio-polymer solutions. As a preliminary to this, it was decided to attempt to devise more direct methods of measuring this rate than those used by Wall and his coworkers. Two such methods were devised and tested in polyelectrolyte solutions similar to those studied by Wall.

The first method employs the recently developed glass electrodes whose potentials (with respect to a suitable reference electrode) indicate the activities of certain cationic species, even in the presence of other cations. Using Na-polyacrylate solutions as an example, the activity of Na^+ is first determined with a Na^+ sensitive glass electrode. The quantity bound to polyacrylate is then estimated as the difference between the actual concentration of Na^+ ions in the solution, and the activity calculated from the electrode measurement. An electrolyte, such as NH_4Cl, whose cation does not affect the potential of the glass electrode is then added. As a result of the competition between NH_4^+ and Na^+ ion for the "binding" sites of the polymer, there is an increase in free Na^+ in the solution. The potential of the sodium sensitive electrode changes in response to this increase in free sodium concentration. The rate of exchange between added NH_4^+ ions and bound Na^+ ions can then be calculated from the rate of change of the potential with time. This method has several limitations, the most important, for our purposes, stemming from the slow time of response of the glass electrode to changes in activity of Na^+ ion, about 2 minutes for 99% of a potential change.

The second method involves the rate of exchange between Na^+ ions in a cation exchange resin and radioisotopically labelled Na^+-polyacrylate solution. It is known that with well stirred dilute solutions, the rate of exchange between ions in an ion-exchange resin and those in solution is proportional to the concentration of ions in solution; this is a consequence of the control of this rate by the diffusion of ions across an unstirred thin layer of solution surrounding the resin particle. Since large, highly charged negative ions, such as polyacrylate ion, cannot enter a cation-exchange resin, Na^+ ions which are "bound" to the polymer ion will remain in the solution phase. Accordingly, the experimentally observed rate of uptake of radioactive Na^+ ions by the resin would be expected to be controlled by the concentration of "free" radioactive Na^+ ions in the solution. A slow rate of exchange between bound and free ions would result in rapid uptake of Na^+ ions which are initially free, followed by a slower uptake of Na^+ at a rate limited by the rate of exchange of radioactive Na^+ ions bound to the polyacrylate for free non-radioactive Na^+ ions. The rate of exchange between free and bound Na^+ ions would therefore be calculable from the change in the slope of a rate of exchange vs. time curve.

Major Findings:

Over a wide range of conditions, the exchange between free and bound Na^+ ions in Na^+ polyacrylate solutions was found to be completed within the resolving times of the two methods, about 1 minute in the case of the Na^+ sensitive electrode technique, and about 1/2 second with the ion-exchange resin method. Since these results were not considered to be compatible with those reported by Wall et al., some of the experiments of these workers were repeated with our sample of polyelectrolyte, and good agreement with their data was obtained. Subsequent experiments, based on modifications of the methods employed in Wall's transference experiments, indicated that these experiments are accompanied by extraordinarily large electro-osmotic effects. Although these effects could seriously affect the rates of ion movement, they were not considered by Wall et al. in the interpretation of their data. It appears, therefore, that the conclusion of these workers, i.e. of extremely slow rates of exchange between free and bound Na^+ ions in polyelectrolyte solutions, is fallacious.

Significance to NIAMD Research:

The question of whether small ions, such as the alkali metal and halide ions, are "bound" to polyelectrolytes in a kinetic sense, is important in regard to understanding the mechanism of ion movement across both artificial and biological membranes, which may have ionogenic groups. This question is also important for understanding ion movement within cells, in which there is a high concentration of charged macromolecules.

A large part of the interpretation of titration curves of proteins has been based on the unproven assumption that certain small ions compete for "binding" sites on the protein. The part of the work of Wall concerning the net movement of Na^+ ion to the anode in electrical transference measurements has been cited in the literature as one of the strongest arguments, by analogy, for such specific site binding. It is very possible that the interpretation of this aspect of the transference data is also complicated by electroosmosis.

Proposed Course of the Project:

The results reported in this Report are being prepared for publication. An attempt will be made to devise methods for eliminating the effects of electroosmosis in the measurement of electrical transference properties of charged macromolecules.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LPB-10
1. Physical Biology
2. Electrochemistry and
   Colloid Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The direct measurement of the forces of attraction and repulsion between plates, lenses, microbeads of defined geometry.

Previous Serial No.: NIAMD-LPB-9

Principal Investigator: Karl Sollner

Other Investigators: Melvin H. Gottlieb

Cooperating Units: None

Man Years:

Total:	1/4
Professional:	1/4 (MHG 1/4, K.S. None)
Other:	None

Project Description:

Objectives:

To measure directly long range forces of attraction and repulsion between macroscopic objects in aqueous solutions. We have only considered repulsive forces in our initial studies.

Methods Employed:

The methods used were essentially the same as described in last year's Report. Microscopic spheres are allowed to settle onto a smooth surface in various media, and the equilibrium distances between the spheres and the surfaces are measured using a visible light interference technique.

Major Findings:

As mentioned in last year's Report, our efforts were centered on systems with polystyrene spheres and glass plates. However, despite some initial encouraging results with these systems, it was not found possible to obtain results which were reproducible enough to even qualitatively indicate the existence or absence of long range forces between these objects. It has become clear that the facilities and manpower which would be necessary for significant progress on this problem are far beyond our original estimates.

Proposed Course of the Project:

Since we were not able to obtain reproducible results after two years' work with a number of systems and with a number of modifications of our experimental methods, work on this project was discontinued as of approximately October 1, 1967.

Significance to NIAMD:

The direct measurement of long range forces between interfaces would have had an important bearing on understanding the formation and stability of both tissues and of microstructures within cells.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LPB-11
1. Physical Biology
2. Electrochemistry and
   Colloid Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The physical chemistry of membranes and complex membrane systems of biological interest

Previous Serial Number: NIAMD-LPB-10

Principal Investigator: Karl Sollner

Other Investigators: Gerald M. Shean, Melvin Gottlieb, Vedoster Ingram

Cooperating Units: None

Man Years:

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives:

The physico-chemical study of liquid ion-exchange membranes, permselective ion-exchange membranes, other membrane systems and models with the ultimate aim of providing a rational basis for various complex membrane phenomena in living systems.

Methods Employed:

The methods employed were essentially the same as reported previously, including flamephotometry, potentiometric titrations (including the chloridimeter), spectrometry, nonaqueous titrations, radioactive tracer methods, etc.

Major Findings:

Liquid ion exchanger membranes prepared from about twenty different quaternary (strong base) ammonium compounds dissolved in a variety of organic solvents were screened for their electromotive behavior in cells:

Solution 1	Liquid membrane	Solution 2
0.1 N KI		0.2 N KI

Eight membranes containing one of these compounds, including both aliphatics and aromatics, yielded concentration potentials which approach the theoretical maximum within a few tenths of a millivolt. For more detailed investigation, membranes based on trioctyl propyl ammonium in the form of various halides were chosen. These compounds are soluble in a number of organic solvents, and have only slight water solubilities and high solvent-water distribution coefficients. Membranes based on a variety of solvents, with dielectric constants between 2.7 and 27, gave concentration potentials close to the theoretical maximum. There is no correlation between the dielectric constants of the various solvents and the deviations of the concentration potentials from the theoretical maximum. The same holds true for the specific resistances of the membrane phases. With suitable solvents, the electromotive properties of the membranes are independent of the concentration of the active compound in the membrane phase. For instance, membranes consisting of o-dichlorobenzene as solvent and trioctyl propyl ammonium chloride in concentrations of 0.1% (resistance $> 3 \times 10^6 \Omega$) up to 100% yielded near-maximum concentration potentials. At medium solution concentrations there is not much difference between cells with the various halides; at low concentrations, however, as it was to be expected, ions high in the Hofmeister series, like I^- and CSN^-, show only small discrepancies between calculated and measured potentials. For instance, a 20% trioctyl propyl ammonium iodide in o-dichlorobenzene membrane gave virtually theoretical concentration potentials, within the limits of the experimental error, in the concentration range from 1.0 N KI all the way to .002 N KI. Also in agreement with expectation, the strong base trioctyl propyl ammonium halide membranes yield considerably higher concentration potentials at low solution concentrations than do the weak base membranes reported on in previous years.

The search for basic organic ion exchangers based on multivalent atoms other than nitrogen led to several compounds that resembled the quaternary ammonium bases: tetrabutyl phosphonium bromide, tetraphenyl arsonium chloride, tetraphenyl antimony bromide, triphenyl selenium chloride, and phenyl mercuric chloride. When dissolved in o-nitrotoluene and used as membrane between the appropriate 0.1 N and 0.2 N solutions, only the tetraphenyl antimony bromide membranes gave a concentration potential which reached the maximum theoretical potential, within several tenths of a millivolt.

Permselective (porous) membranes. Work was completed on the paper, mentioned in last year's Report, concerning the anomalous relationship found between the electrical resistances and rates of self-exchange of ions across certain permselective collodion matrix membranes with a quaternary ammonium base as electrochemically activating compound. In the course of a systematic attempt to elucidate the observed anomaly we determined the influence of several parameters on the magnitude of the discrepancy between theoretical and the experimental relationship between membrane resistance and rate of self-exchange. The magnitude of this discrepancy is independent of temperature in the range 5°C. to 25°C., and of the pH of the solutions separated by the membrane in the range 2 to 10.

Experimental data (using radio tracers) on the relationship between the rates of self-exchange $A \leftrightarrows A^*$ and $B \leftrightarrows B^*$, and the rate of the interexchange $A \leftrightarrows B$ of different ions across permselective membranes are being prepared for publication. A theoretically predictable relationship was confirmed with neutral solutions of several strong electrolytes, but failed in the case of H^+ as one of the interexchanging ions.

Most of the time of the senior investigator was spent on critical reviews of some general topics in the field of membranes. As reported last year, a comprehensive and critical survey of the widely scattered background information on "Liquid membranes" is in preparation at the request of the organizers of a NATO Advanced Study Institute on "Synthetic Polymer Membranes". Substantial progress in this direction was made during the last year. Similarly, a reviewlike paper on "The Electrochemistry of Porous Ionic Membranes" (the title may still be changed) was requested by the organizers of a meeting sponsored by the California Polymer Chemistry Section of the American Chemical Society and a number of biological investigators. The purpose (and the tenor) of this paper, as that of the whole meeting and the planned Symposium Issue of Macromolecular Science is to stimulate polymer chemists to apply their specialized knowledge to biological membrane problems, and biologists to look to polymer chemistry as a source of much pertinent information. A lengthy paper trying to serve this purpose was nearly finished at the time this Report was written.

Proposed Course of the Project:

Liquid ion exchanger membranes. Extension of the measurement of concentration potentials in cells with trioctyl phenyl ammonium salt membranes to a great variety of potential determining ions over wide ranges of solution concentrations. Obtaining more complete bi-ionic potential data. Continuing the search for better liquid cation exchangers. Expanding the study of liquid anion exchange membranes containing compounds based on multivalent atoms other than nitrogen.

Permselective (porous) membranes. Continuation of the efforts to understand the anomalous relationship between the electrical resistances and rates of self-exchange of ions across quaternary ammonium type permselective membranes. After a study of the effects of various additional parameters such as charge density of the activating polyelectrolyte will be completed, it is planned to make a detailed survey of the electrical impedance characteristics of these membranes, with particular emphasis on extremely short d.c. pulses. Measuring the effects of pH and membrane hydrolysis on concentration potentials across permselective membranes at low solution concentrations.

Significance to NIAMD Research:

Movement of solvents and solutes is an important aspect of all life processes. This project tries to provide solidly founded, nonspeculative physico-chemical background information on the functional behavior of membranes in general. At the same time, these membrane studies have furnished a new, now widely used tool for the electrometric determination of ion activities--the use of permselective membranes as membrane electrodes. It is perhaps also of interest that Sollner's "permselective" membranes are the original prototypes of the ion exchange membranes which are used now in the electrodialytic desalination of sea and brackish water. The work of recent years, such as model studies on the accumulation of electrolytes against concentration gradients and on carrier transport, and the successful prediction of certain involved effects in multi-ionic membrane systems, indicates that an increasing variety of complex membrane effects, similar to those in living nature, are slowly becoming amenable to a fairly complete physico-chemical analysis.

Honors and Awards: None

Publications:

Sollner, K.: Membrane Electrodes. Annals N. Y. Acad. Sci. 1968 148, (1) 154-179.

Sollner, K. and Shean, G.: Liquid Ion Exchange Membranes of Extreme Ionic Selectivity and High Transmissivity. Protoplasma 1967 63, (1-3, Proceedings of the Symposium on Biophysics and Physiology of Biological Transport, Frascati, June 1965) 174-180.

Gottlieb, M. H. and Sollner, K.: Failure of the Nernst-Einstein Equation to Correlate Electrical Resistances and Rates of Ionic Self-Exchange Across Certain Fixed Charge Membranes. Biophys. J.: In Press, May 1968 issue.

Serial No. NIAMD-LPB-12
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:

1. Determination of the structural characteristics and kinetics of the formation of complexes between phospholipid (ethanolamine glycerophosphatide and serine glycerophosphatide) and their possible role in the rhodopsin molecule.

2. Further investigation into the composition of lipid extracts of rhodopsin and how it varies under the influence of light, changes in pH and sequence of extraction procedures.

Previous Serial Number: NIAMD-LPB-11

Principal Investigator: Ralph G. Adams

Other Investigators: None

Cooperating Units: None

Man Years

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives:

1. Under conditions of exclusion of water, H^+ and oxygen, ethanolamine glycerophosphatides (PE) and serine glycerophosphatides (PS) will complex with retinal (the chromophore molecule of rhodopsin) to form colored products whose λ maximum falls between 460 and 485 nm. These complexes form also from the extracted components of rhodopsin in the extracting material provided the proper conditions exist, however, it is more convenient to use pure materials.

   By following the increase in absorption of a mixture of PE (for example) and 11-cis retinal in chloroform in the dark in the Cary, one obtains a series of spectra which can be examined on the curve analyser and the time course of complexing can be determined. Using an iterative technique it is also possible to obtain absorption coefficients for the complexes.

   NMR and IR give some evidence as to structure.

2. Although the phospholipids of rhodopsin have been identified and isolated (1966-67 report) some doubt remained as to whether all were integral parts of the rhodopsin molecule and it seemed essential to further pursue this question by attempting to purify rhodopsin to the ultimate to see whether any difference occurs. This seemed best accomplished by using all the reported techniques such as column chromatography, ion exchange, sacrificial centrifugation (taking only the small part isolated by minimal disruption of the retinal rods) and great precaution to avoid deterioriation by light or heat (fast trips to the abattoir for freshly excised eyes).

Major Findings:

1. The greatest complexing activity is between PE and retinal with a λ maximum at 478 nm (1). However, there are two additional peaks formed at about 463 (2) and 470 nm (3). The (1) peak has the fastest formation time and seems to be about twice as fast as (2). Peak (3) starts forming only after (1) levels off. The iterative processes require many samples and many spectra and are not complete.

   The complexes appear to be Schiff's bases since they are affected by hydrogen ion, being destroyed by excess and since this is the most logical combination between an aldehyde and a molecule with an amino group.

2. After many preparations have been made and examined by methods previously described, no purification method changed the chloroform-methanol fraction which remains at about 28 moles phospholipid per mole rhodopsin. The ethanol extract on the other hand, as suspected, probably contains fortuitous lipids extracted by digitonin from the retinal rod membranes along with the rhodopsin, and varies in composition and amount in proportion to the care exercised and the "purity" of the rhodopsin preparation. Column chromatography, ion exchange resins and sacrificial centrifugation techniques were not as effective as extreme freshness of the eyes used to supply rhodopsin.

Significance to NIAMD Research:

1. Although many structures have been proposed and various amino acids purported to be the binding site of retinal in rhodopsin--none have been demonstrated "in vitro" and no other complex with retinal exhibiting λ maximum anywhere near 500 nm has involved a natural substance found in the retina. It is possible PE could account for the color of rhodopsin whose spectral shift has been a source of much debate.

2. Before one can embark upon a project in which an attempt to construct a membrane similar to the retinal rod membrane, obviously a good estimate of the composition of the natural product must be made. The criterion

of purity of rhodopsin has never been firmly established, thus there is no basic information by which one can measure composition. I feel the exhaustive examination I have made does set a lower limit.

Honors and Awards: None

Publications: Adams, Ralph G.: Effect of Light on Extraction of Lipid from Retinal Rods. J. Lipid Res. 8:245, 1967.

Serial No. NIAMD-LPB-13
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Molecular structure determined by spectroscopic methods

Previous Serial Number: NIAMD-LPB-12

Principal Investigator: Edwin D. Becker

Other Investigators: Robert B. Bradley
Regitze R. Shoup
Livio Paolillo
Kenneth Saum

Cooperating Units: H. T. Miles
J. Ferretti (DCRT-5.5)

Man Years

Total:	5
Professional:	4
Other:	1

Project Description:

Objectives:

(1) Development of an understanding of the forces within and between molecules, especially those of potential biological importance.

(2) Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Methods Employed:

The primary techniques used in this work are infrared spectroscopy (IR), Raman spectroscopy, and nuclear magnetic resonance (NMR). The three methods supplement each other in providing detailed information about the structure of molecules. In addition, the spectra are highly sensitive to the effects of molecular interactions.

Major Findings:

(1) Nuclear magnetic double resonance (NMDR) has been used to determine ^{15}N chemical shifts in isotopically enriched compounds by studying the resonance of proton spin coupled to the ^{15}N. The advantages of the double resonance technique relative to ordinary NMR are an enhancement of

sensitivity (by a factor of 1000 for ^{15}N) and an increase in accuracy of frequency measurement. Initial studies of hydrogen bonding by this method have shown that the ^{15}N chemical shift is affected only slightly in the proton donors aniline and methyl amine but markedly in the proton acceptor quinoline (Paolillo and Becker).

(2) NMDR has also been applied to the determination of the relative signs of coupling constants of methylamine-^{15}N (Paolillo and Becker) and of methyl and ethyl isocyanide (Paolillo and Ferretti).

(3) The Nuclear Overhauser effect (a type of NMDR experiment) has been found to be applicable to the location of substituents in molecules of the Elsinochrome type (Paolillo and Lousberg).

(4) NMR studies of 1,7,7-trimethylcytosine have shown a pronounced solvent dependence of the rate of internal rotation of the dimethylamino group. The presence of a ribose moiety in place of the 1-methyl group also alters the internal rotation rate of the $N(CH_3)_2$ (Shoup, Miles and Becker).

(5) The Cary laser-Raman spectrometer has been in operation since September, with considerable effort going toward the determination of optimum conditions for various types of studies. A careful analysis of depolarization ratios in p-benzoquinone and its completely deuterated analog has provided information pertinent to a refinement of the vibrational assignment of these and related molecules and, moreover, has established a useful working relationship between two different methods of determination of depolarization ratios (Saum and Becker).

Significance to NIAMD Research:

In this project we are attempting to obtain basic information on the structure and molecular interactions of nucleosides and their derivatives and to learn more about the nature of hydrogen bonding in these and other molecules. Such information may aid in an understanding of the relation between structure and function of nucleic acids and enzymes. In addition, the development of spectroscopic techniques applicable to molecules of biological activity and interest, such as steroids, alkaloids, pigments, etc., aids in the structure elucidation of many natural and synthetic substances studied throughout NIH.

Proposed Course of Project:

(1) NMDR will continue to be applied to the study of ^{15}N resonances, first to complete the study of hydrogen bonding effects and later to study internal rotation in nucleoside derivatives and in certain nitroso compounds.

(2) Quantitative measurements will be made of the rate of internal rotation of amino and substituted amino groups in nucleoside derivatives. These studies will utilize ordinary proton NMR and pulsed NMR.

(3) The completion of Raman studies of p-benzoquinone and its isotopic derivatives will provide the basis for a refinement of the vibrational assignment and force constant determination for this interesting series of molecules.

(4) A new study will be started in the fall on the vibrational assignment of uracil and related molecules. The objective is the determination in detail of the modes of vibration of this molecule in order to be able to interpret IR data obtained by Miles on the interaction of natural and synthetic polynucleotides.

Publications:

Shoup, R. R., Miles, H. T., and Becker, E. D.: Steric control of geometric isomerism in cytosine cations. _J_. _Am_. _Chem_. _Soc_. 89: 6200-6204, Nov. 1967.

REPORTED IN PRESS - 1967

Sheinblatt, M., and Becker, E. D.: Spectroscopic studies of copper (II) complexes of glycylglycine. _J_. _Biol_. _Chem_. 242: 3159-3163, July 1967.

Serial No. NIAMD-LPB-14
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Molecular structure, organization and intermolecular forces

Previous Serial Number: NIAMD-LPB-13

Principal Investigator: Elliot Charney

Other Investigators: William Eaton
Julie Milstien
Kiwamu Yamaoka

Cooperating Units: Professor Robert M. Hexter, Department of Chemistry,
Carnegie-Mellon University
Dr. Lin Tsai, NHI-LB
Dr. Ruth McDiarmid, NIAMD-LPB-18

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

The general objectives of this research are to determine the role of molecular structure and of intermolecular forces in chemical, physical and biological phenomena by exploring the electrical (dipole moment and dielectric polarization) and optical (spectroscopic absorption, optical rotatory dispersion, dichroism) properties of molecules.

Methods Employed:

Except for one technique, the methods used are largely standard spectroscopic techniques. The measurements of electric field induced dichroism are, however, unique. This procedure involves measuring the ultraviolet absorption of a solute, parallel and perpendicular to a pulsed high voltage field.

Major Findings:

1. Optical activity of conjugated chromophores.

   a. An entirely new investigation of the optical activity of the conjugated diketone, camphorquinone, has been started. This was undertaken to answer two major questions, but the experimental results are revealing a host of new interesting problems. The questions are: (1) to what extent are two ketone chromophores coupled in diketones and what does the extent of the coupling tell us about the electronic structure of diketones in the ground and excited states; and (2) how good is modified Hückel molecular orbital theory in predicting the optical and spectroscopic properties of this and related systems. Camphorquinone is the molecule of choice because of its rigid structure, because it can be prepared in stereochemically pure from d(+) camphor, and because the s-cis configuration of the diketone makes symmetry considerations helpful in the analysis. All these properties make it ideal with respect to the use of optical activity and/or circular dichroism as a tool in the analysis, while the first and third are important from the point of view of the use of absorption and emission spectroscopy as tools. Our results, which are still incomplete, already show that there is extensive coupling between the electronic transitions with respect to the corresponding isolated mono-ketone for example in d(+) camphor. We are also finding from the vibrational spectra (infrared and Raman) that the diketone is behaving in this complex molecule as though it were unaware of the lack of symmetry in the remainder of the molecule.

b. The diketone, benzil, like camphorquinone and the simple molecules glyoxal and biacetyl, is representative of a large class of conjugated diketones. In benzil, however, in addition to the other questions, we are interested in knowing whether the optical activity of the crystal comes primarily from the inherently optically active benzil molecule (which is however, optically inactive in solution because of rapid racemization around the single bond between the carbonyl groups) or from the induced activity due to the screw symmetry of the molecules in the unit cell of the crystal. We have thus far successfully measured the circular dichroism of both single crystals and of micro crystals embedded in KBr discs using the new technique described in the previous annual report.

2. Ultraviolet spectra of ethylene.

Our investigation of the far-ultraviolet spectrum ethylene is discussed in Dr. McDiarmid's report.

3. Electric field induced dichroism of large molecules.

a. DNA: The difference in absorption of polarized light parallel and perpendicular to the electric field used to orient DNA molecules has given some unpredicted results, namely, that the field dependence of the dichroism ($\Delta\varepsilon = \varepsilon_{\parallel} - \varepsilon_{\perp}$) has a large linear component, although it was expected that it would be quadratic. In order to determine whether this results from a distortion of the molecule in the large fields of 700 volts/cm to 8000 volts/cm, we have sonicated DNA by established procedures to half million molecular weight and are in the process of making electro-dichroism on the sonicated DNA.

Even more interesting perhaps is the wave length dependence of the electro-dichroism of DNA which is showing the effect of a weak band on the long wave length edge of the 259 mμ absorption band which may be the elusive $n \rightarrow \pi^*$ band and which is indicative of a small twist of the DNA bases relative to each other or relative to the helix axis. We hope to examine this more carefully on the sonicated DNA which, from information obtained from the light scattering data of Eysenberg and others, should be more rod-like.

b. Polypeptides: Preliminary measurements of the electric field induced dichroism of poly-γ-benzyl-L-glutamate indicate that despite the fact that the phenyl ring is separated from the optically active peptide carbon atom by six essentially single bonds of which 3 present little or no hindrance to rotation, the phenyl rings are oriented with respect to the polypeptide. In view of this, a search was instituted for circular dichroism due to the 260 absorption bond (the optical activity has been measured by Doty, Blout and others and no activity from this band has been found). The CD distinctly shows the presence of this band, and the sign of the optical activity follows the sense of the helix axis rather than of the optically active center as determined by measurements on the poly-γ-benzyl-D-glutamate and poly-β-benzyl-L-aspartate.

Significance to NIAMD Research:

Using the probe of electron-magnetic radiation to determine some of the optical and electrical properties of small molecules, information is obtained to help elucidate the structure and forces between both small and large molecules.

Proposed Course of Project:

The electro-dichroism and birefringence data on DNA, both the native molecule and the native sonicated molecule, will be analyzed to obtain structural parameters for DNA in solution. The poly-benzyl-glutamate studies will be continued and extended to other molecules. These results should enable us study conformational changes under dynamic conditions. To pursue these, we will need to modify the apparatus slightly to enable us to obtain higher signal to noise by singal averaging techniques.

We will continue to study the ultraviolet spectra of olefins in line with our original aim of understanding the spectra well enough to be able to use them to help write good enough molecular wave functions to describe and/or predict the optical and physical properties of the double bond.

The optical activity of conjugated chromophores in solution and solids will be pursued as part of the same effort described with respect to olefins. One aspect of this, not mentioned above, namely examination of the extraordinarily interesting emission spectra we have observed for camphorquinone, will be pursued for the meaning it has in the geometry of the excited states of these molecules and the theory of internal conversion of energy between excited states.

Publications:

REPORTED IN PRESS - 1967

Charney, E., and Bernhard, S. A.: Optical properties, and the chemical nature of acyl-chymotrypsin linkages. J. Am. Chem. Soc. 89: 2726-2733, May 1967.

Ziffer, H., Weiss, U., and Charney, E.: Optical activity of non-planar conjugated dienes. IV. Interacting chromophores in gliotoxin. Tetrahedron 23: 3881-3886, Oct. 1967.

McDiarmid, R., and Charney, E.: The far ultraviolet spectrum of ethylene and ethylene-d_4. J. Chem. Phys. 47: 1517-1524, Aug. 1967.

Serial No. NIAMD-LPB-15
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Physical and Chemical Bases of Photoreception

Previous Serial Number: NIAMD-LPB-14

Principal Investigator: W. A. Hagins

Other Investigators: Robert E. McGaughy
Frances M. Hagins
Richard D. Penn

Cooperating Units: Erhard Gross, John M. Tormey and Kurt Heinrich.
Marine Biological Laboratory, Woods Hole, Massachusetts.
U. S. National Bureau of Standards.

Man Years

Total:	3 1/4
Professional:	2 1/4
Others:	1

Project Description:

Objectives:

To outline the successive events by which light quanta absorbed in the receptors of animals lead to the transmission of information to the brain.

Methods Employed:

1. Measurements of currents, voltages, and impedances of the retina by conventional electronic methods.
2. Measurement of photochemical changes in rhodopsin in living cells by kinetic microspectrophotometry.
3. Analysis by digital computation of electrical noise produced by photoreceptors.
4. Investigation of the chemical structure of rhodopsin by spectrophotometry and amino acid analysis.
5. Electron microscopy of the cellular structure of photoreceptors.
6. Analysis of the ionic composition of the retina by electron probe microanalysis.

Major Findings:

1. Direct microelectrode recording techniques have shown one component of the fast photovoltage of the squid retina to be due to a localized charging of the electrical capacity of the cell membranes in the outer segments of the photoreceptors. The current responsible for the charging process begins without detectable delay and is directly proportional to the rate of decay of lumi-rhodopsin, a highly unstable intermediate substance in the photolysis of rhodopsin. Thus it is believed that rhodopsin and its photochemical decomposition products are closely associated with the capacitative part of the plasma membranes of the photoreceptors and are probably a part of the membrane structure.
2. Three electrical methods have been used to measure the electrical cable constants of squid photoreceptors, one based on externally applied currents, one on the locally excited physiological receptor current and one on the fast photovoltage. By all three methods, the cable constants are essentially the same, thus indicating that the same cellular structures that govern the passive electrical properties of the squid retina are also the source of both the fast photovoltage and the receptor current. The microvilli which are a conspicuous feature of the outer segments apparently are out-pouchings of the cell membranes and serve to increase their surface areas by a factor of nearly 20 over those of smooth cells of similar size.
3. The heat produced when light is absorbed by squid rhodopsin has been measured directly by calorimetry. Light is apparently quantitatively converted to heat by the photopigment. Moreover, the resulting heat diffuses through the retina at essentially the same rate as through liquid water.

Significance to NIAMD Research:

Progress in the understanding of molecular events underlying excitation of photoreceptors may provide a new point of attack on the nature of permeability changes which occur in the surface membranes of electrically active cells, such as nerve and muscle, during transmission of nerve impulses and initiation of muscular contraction.

Proposed Course of Project:

The immediate aims of our research are (1) to find independent evidence for structural changes in rhodopsin which may underly the fast photovoltages in the squid retina, and (2) to develop a means of studying the permeability changes responsible for the physiological photocurrent of squid photoreceptors in isolated, non-living retinal material. The ultimate aim is a molecular explanation of light's ability to produce specific membrane permeability changes in the cell membranes of photoreceptors.

Publications:

Hagins, W. A., and McGaughy, R. E.: Molecular and thermal origins of fast photoelectric effects in the squid retina. Science 157: 813-816, Aug. 1967.

Hagins W. A., and McGaughy, R. E.: Membrane origin of the fast photovoltage of squid retina. Science 159: 213-215, Jan. 1968.

Serial No. NIAMD-LPB-16
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Structure of paramagnetic molecules and their interactions with their environment

Previous Serial Number: NIAMD-LPB-15

Principal Investigator: Hideo Kon

Other Investigators: Nobuko Kataoka

Cooperating Units: Herman Ziffer (NIAMD-LPB-23)
Ulrich Weiss (NIAMD-LPB-22)
Robert Lousberg

Man Years

Total: 2
Professional: 2
Other: 0

Project Description:

Objectives:

To increase our knowledge of the electronic structures of paramagnetic small molecules, and to apply it to the understanding of their function in biological systems.

Methods Employed:

Electron spin resonance (ESR) was the basic means of the observation. For analysis the simulated spectrum was constructed by use of a digital computor. The computor was also used to calculate spin density distributions and for quantitative intensity measurement of ESR absorption.

Major Findings:

1. Further study of nitric oxide hemoglobin by ESR (cf. previous report) revealed that the dehydration of the protein by evacuation causes a change in the ESR absorption quite similar to that caused by Na-dodecylsulfate (SDS); i.e. the overall low field shift of the absorption and the development of the N^{14}-hyperfine splitting in the high field part of the absorption. This result points to the close

similarity of the effect of SDS and the protein dehydration on the structure surrounding the heme group.

2. The interaction of 0.03 ∿ 0.5 M Na-salicylate with nitric oxide-hemoglobin was found to change the ESR absorption from one characteristic of NO-hemoglobin to a new group of absorption. The latter can be interpreted as arising from some low-spin type Fe(III) system. Above 0.5 M, the original spectrum is recovered, but with some modification such as to indicate the structural randomization around the heme group. Significantly, Na-salicylate, in the above mentioned concentration range, is known to cause a marked enhancement in oxidase-like activity (O_2 up-take) of O_2-hemoglobin. Thus the change of ESR absorption appears to have an interesting bearing on the change of hemoglobin functioning.
3. (With Dr. Kataoka):
   a. It was found that the reaction of phenylisocyanide with pentakis-(phenylisocyanide)Co(II) perchlorate at -50°C yields an unstable paramagnetic species which was proved by ESR to be hexakis-(phenylisocyanide)Co(II) perchlorate, a hitherto unidentified truly hexacoordinated low spin Co(II) complex molecule.
   b. From the ESR spectra of the low spin tetrakis(phenylisocyanide)-Co(II) halide, the fractional spin transfer from Co to the ligand halogens was estimated to be about 10% per halogen ion. The observed rather unusual positive isotropic hyperfine coupling between the electron and ^{59}Co nuclear spin indicates that the unpaired electron in d_{z^2} orbital also spends a small fraction of time in the outer s-orbital(s) of Co.
4. (With Dr. H. Ziffer):
   Anion radical of several quinone photodimers were prepared and studied by ESR. The unpaired electron was found to be localized in only one moiety of the dimer when they have "anti" configurations.
5. (With Dr. U. Weiss and Dr. R. Lousberg):
   Study by ESR of the anion radical of several elsinochrome derivatives showed that two-OH protons are involved in the observed hyperfine structure, in addition to the six out of twelve methyl protons. Positive proof of the structure by this method was found difficilt, mainly because of the non-planarity of this π-electron system, a factor difficult to be taken into account in the molecular orbital spin density calculation.

Significance to NIAMD Research:

Accurate information on the electronic states of transition metal complexes is important not only for understanding the behavior of the metal ions naturally occurring in biological systems, but also for using the transition metal ions as an artificial label to probe more complicated systems.

Proposed Course of Project:

The nitric oxide hemoproteins will be further investigated in conjunction with some of the non-heme iron proteins of biological interest. The study of small paramagnetic complexes will also be continued with some emphasis on theoretical interpretation of ESR results.

Publications:

Nishinaga, A., Cahnmann, H. J., Kon, H., and Matsuura, T.: Model reactions for the biosynthesis of thyroxine. XII. The nature of a thyroxine precursor formed in the synthesis of thyroxine from diiodotyrosine and its keto acid analog. Biochem. 7: 388-397, Jan. 1968.

Kon, H.: Paramagnetic resonance studies of nitric oxide hemoglobin. J. Biol. Chem. In press.

Kataoka, N., and Kon, H.: A hexacoordinated low spin Co(II) complex. J. Am. Chem. Soc. In press.

REPORTED IN PRESS - 1967

Kon, H., and Sharpless, N. E.: Nature of the unpaired electron orbital in some low spin oxygen-Co complexes. Spectroscopy Letters 1: 49-54, Jan. 1968.

White, F. H., Jr., Riesz, P., and Kon, H.: Free radical distributions in several γ-irradiated proteins as determined by the free radical interceptor technique. Rad. Res. 32: 744-759, Dec. 1967.

Nishinaga, A., Kon, H., and Cahnmann, H. J.: Model reactions for the biosynthesis of thyroxine. XI. The nature of a free radical formed in the autoxidation of 4-hydroxy-3,5-diiodophenylpyruvic acid. J. Org. Chem. 33: 157-162, Jan. 1968.

Serial No. NIAMD-LPB-17
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Molecular Dynamics

Previous Serial Number: NIAMD-LPB-16

Principal Investigator: Ira W. Levin

Other Investigators: None

Cooperating Units: S. Abramowitz and J. Comeford, National Bureau of Standards
D. F. Eggers, Jr., University of Washington.
W. F. Edgell, Purdue University.

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

Determination of reliable vibrational normal coordinates and molecular charge distribution gradients for polyatomic systems.

Methods Employed:

Various infrared and laser Raman spectroscopic techniques provide the required information for the vibrational analyses. Related properties, such as the vibrational angular momenta, amplitudes of vibration, isotopic frequency differences, and centrifugal distortion effects, contribute to the specification of unambiguous potential functions. The digital computer provides computational aids for the theoretical spectroscopic analyses.

Major Findings:

1. Rotation-vibration zeta parameters, determined from band contour measurements, uniquely specified potential functions for the degenerate modes of PF_5 and AsF_5. The normal coordinate analysis for these bipyramidal molecules unambiguously assigns the axial bending mode at a higher frequency than the in-plane bending motion and suggests a

trend for the assignment of these vibrations in XY_5 molecules. Comparisons between the experimental and calculated mean vibrational amplitudes indicate an insufficient sensitivity of these parameters for limiting the interaction force constants of PF_5 as narrowly as the rotation-vibration zeta parameters. The vibrational amplitude calculation, however, is sufficiently sensitive for distinguishing between the possible assignments for the bending modes. (Levin.)

2. Unique force fields were determined for SiF_4 using the Coriolis zeta constant for ν_3 and the isotopic shift information for ν_4 to constrain the potential function. The Coriolis information was obtained from low temperature gas-phase band contours. A detailed comparison between the two constraints indicates that nearly the same uncertainty is involved in both methods for specifying the stretch-bend interaction force constant, despite the much greater precision inherent in determining isotopic splittings. (Levin and Abramowitz.)
3. An examination of solid and solution Raman spectra of hexachlorobenzene (C_6Cl_6) indicates that the chlorine bonds are inclined to the plane of the benzene ring (as compared to the complete planarity configuration for C_6F_6). Comparisons of the solid Raman spectra of C_6Br_6 and C_6I_6 to the solid C_6Cl_6 spectra also suggests a corrugated model for these two species. (Levin and Abramowitz.)
4. A tentative vibrational analysis is proposed for the o-, m- and p-dicyanobenzene species. Complete solution and solid infrared and Raman data were obtained. (Levin, Abramowitz and Comeford.)

Significance to NIAMD Research:

Since force constants are strongly characteristic of their associated chemical groups, reliable normal coordinates determined from small molecules provide an insight to chemical binding properties of larger molecular species.

Proposed Course of Project:

1. Absolute infrared intensity measurements of the partially deuterated methyl halides are continuing.
2. The use of band contour measurements for providing absolute infrared intensity parameters is being investigated. CF_4, a spherical top molecule, serves as the model system. (Levin and Edgell.)
3. Raman spectroscopic investigations on organic single crystals are anticipated. The polarization properties of the laser source, with a knowledge of the crystal structure, should provide a useful means for assigning vibrational spectra.

Publications:

Levin, I. W., and Abramowitz, S.: Force field for silicon tetrafluoride. J. Res. Natl Bur. Stds. 72A: 156-161, Mar. 1968.

Serial No. NIAMD-LPB-17

REPORTED IN PRESS - 1967

Levin, I. W.: Potential function for phosphorus trichloride. *J. Chem. Phys.* 47: 4685-4690, Dec. 1967.

Serial No. NIAMD-LPB-18
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Electronic and molecular structural investigations.

Principal Investigator: Ruth McDiarmid

Cooperating Units: Arnold M. Bass, National Bureau of Standards
Morris Krauss, National Bureau of Standards
Elliot Charney, NIAMD-LPB-14

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

1. To determine the electronic and molecular structure of molecules predominantly by interpreting their electronic spectra.
2. To explain, by electronic interactions, the spectral differences sometimes observed between molecules that either contain the same single chromophore attached to different non-chromophoric groups or contain more than one of the same single chromophore specifically oriented.

Methods Employed:

1. The measurement and analysis of the ultraviolet absorption spectra of gas phase molecules.
2. The occasional measurement of the infrared absorption spectrum of a specific molecule to assist in the analysis of its ultraviolet spectrum.
3. The calculation, by means of a high speed digital computer, of the electronic energy levels, transition energies, equlibrium geometries and electron distributions of one or two representative molecules.

Major Findings:

1. The vibrational analysis of the $V \leftarrow N$ transition of ethylene (see Charney, 1966-1967) extended to two partially deuterated ethylenes supports our original assignment of the primary vibrational progression as the torsion but provides no explanation for the still

unassigned vibrational interval of 150 cm^{-1}. An extremely weak transition in C_2D_4, predicted to lie to the red of the V←N transition, has recently been located.

2. The still unassigned electronic transition observed in methyl-substituted ethylenes around 200 mμ has been shown by comparison of the spectra of cis- and trans-butene-2 and by determination of the rotational contours of some of the vibronic components of the band in trans-butene-2, to be allowed with an out of plane polarization. The above supports the assignment of this transition as the first member of the ns Rydberg series. A Rydberg series from n=5 to n=10 was found for cis-butene-2 leading to the observed ionization potential of 9.14 eV.
3. An all electron calculation of the ground, $^2E'$ and first Rydberg state of methyl radical has shown that the lowest energy intravalency shell transition lies at 7.83 eV, much higher than anticipated, and that the $^2E'$ state has an equlibrium geometry very different than the ground state. The first Rydberg state was calculated to lie around 5.64 eV, in excellent agreement with the observed vertica transition energy of 5.72 eV.

Significance to NIAMD Research:

An understanding of the electronic origins of the observed spectra of single chromophores and of the spectral changes caused by interactions between either these chromophores or between these chromophores and non-chromophoric portions of the molecule is necessary before observed spectra and spectral changes can be unquestionably used to determine molecular structure. Inasmuch as spectroscopy is widely used in determining molecular structure, the significance of this investigation derives from confidence it gives to spectroscopic determinations of molecular structure.

Proposed Course of Project:

1. The recently located transition in C_2D_4, and an analogous stronger transition in C_2HD_3, will be examined to see if it is the lowest energy forbidden transition predicted by theoretical calculations.
2. Further attempts will be made to find a Rydberg series in trans-butene-2 that includes the 200 mμ band, so it can be assigned unambigiously.
3. Theoretical calculations will be extended to ethylene and to methyl substituted ethylenes to both aid in the analysis of the experimental spectra and to determine the effect of methyl substitution on the energy of and charge distribution along the double bond.

Publications:

REPORTED IN PRESS - 1967

McDiarmid, R., and Charney, E.: Far ultraviolet spectrum of ethylene and and ethylene-d_4. J. Chem. Phys. 47: 1517-1524, Aug. 1967.

Serial No. NIAMD-LPB-19
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanisms of energy transfer at cellular sites of photochemical action.

Previous Serial Number: NIAMD-LPB-17

Principal Investigator: Rodney A. Olson

Other Investigators: William H. Jennings

Cooperating Units: Dr. John M. Olson, Brookhaven National Laboratory
Mr. Charles Hanna, Laboratory of Physical Biology, NIAMD

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

To interpret the mechanisms of energy transfer and allied metabolic steps at cytological sites of photochemical action. To determine the role of chromophore orientation in laminar photoreceptors (chloroplasts) and in crystalline pigment-protein systems and in paracrystalline pigment-lipid systems simulating the organized structure of the photosynthetic apparatus.

Methods Employed:

Modular optical systems for microscopic investigation of living cells permit the study of absorption, emission, polarization and excitation spectra at extremely low light levels. The areas measured range in size from one to ten square microns.

Major Findings:

Optical properties of Bacteriochlorophyll-protein crystals: Correlation of the optical properties of Bchl-protein crystals (Cps. ethylicum) with data from X-ray crystallography and chemical properties permits us to develop a working hypothesis for the Bchl-protein macromolecule. The complex is envisioned as four identical subunits each containing five Bchl molecules associated mainly with the hydrophobic protein residues in the interior of the ellipsoidal macromolecule. The dichroism of the 603 and 809 nm transitions of Bchl indicate that this tetrahydroporphrin is tilted with respect to the optic axis and ring V is positioned in the polar macromolecular surface while ring II is directed toward the hydrophobic macromolecular interior. On the basis of the present work it appears reasonable to postulate that chlorophyll molecules are generally embedded within the protein of macromolecular complexes.

Energy Transfer in a Complex of Rhodamine with Bacteriochlorophyll Protein: Several spectral properties of rhodamine 6G in dyed crystals of bacteriochlorophyll (Bchl) protein indicate the occurrence of a dye:pigment complex. Component analysis of the absorption spectrum of the dyed crystal shows that the oscillator strength of the 527 nm. electronic transition of rhodamine is attenuated to a value of less than one half that of the uncomplexed dye. This attenuation does not occur in dyed crystals from which Bchl has been extracted. Dyed Bchl-protein crystals show a moderate negative dichroism (D'=3.5) in the spectral region of 527 nm. This indicates that the preferential orientation of the 527 nm. rhodamine oscillators should favor energy transfer to the 603 nm. Bchl oscillators as these also exhibit a negative dichroism (D'-1.3). This is confirmed by the observation that rhodamine fluorescence is quenched in Bchl-protein crystals and that excitation energy is transferred to Bchl which fluoresces at 818 nm. No quenching of rhodamine fluorescence occurs in dyed crystals from which Bchl has been extracted. Fluorescence excitation spectra of dyed and undyed crystals confirms the transfer of energy from the 527 nm. rhodamine absorption to the 818 nm. Bchl emission. The possible binding sites of rhodamine can be evaluated in terms of the proposed model of the Bchl protein macromolecule.

Significance to NIAMD Research:

Currently emphasis falls on the orientation of pigment molecules and their role in energy transfer and energy transformation. The optical properties of crystalline porphyrin-protein complexes permit analysis of defined systems leading toward characterization of the active structural site of porphyrin binding within the protein. Since the fine structure of the Bchl-protein complex (Cps. ethylicum) may be closely related to that of the bacterial chromatophore, the study of its role in photochemical processes leads to a better interpretation of energy transfer in all living cells, whether light or chemically activated.

Proposed Course of Project:

1. Continuation of the optical characterization of bacteriochlorophyll in protein complexes including: (a) low temperature microspectrophotometry. (b) Polarization spectra.

2. Study of the yellow fluorescent product developed by photooxidation of chlorophyll in the Hymenomonas pigment body including: (a) Fluorescence excitation spectra to characterize the weak absorption of this product *in vivo*. (b) Kinetics of the spectral changes of fluorescence during photooxidation to elucidate this photochemical reaction.

3. If the material permits, the crystalline chromoprotein phycoerythrin will be studied in the same fashion as was the Bchl-protein complex.

Publications:

Olson, R. A., Jennings, W.H., and Allen, M. B.: Spectral properties of a pigmented body in *Hymenomonas* sp. An extrachloroplast organelle containing chlorophyll. *J. Cell. Physiol.* 70:133-140, 1967.

Olson, R. A., Jennings, W. H., and Olson, J. M.: Chlorophyll orientation in crystals of bacteriochlorophyll-protein from green photosynthetic bacteria. *Arch. Biochem. Biophys.* (in press).

Serial No. NIAMD-LPB-20
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Spectral, physical-chemical and photochemical properties of biologically active substances.

Previous Serial Number: NIAMD-LPB-19

Principal Investigator: Norman E. Sharpless

Other Investigators: None

Cooperating Units: Charles L. Greenblatt, NIAID-LPD-125-0
Hideo Kon, NIAMD-LPB-16

Man Years

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives:

The investigation of the influence of structure of molecules on physical and biological properties, with the object of correlating physical properties with biological activity.

Methods Employed:

Biologically active molecules have been investigated by various forms of spectrophotometry (visible, UV, IR, NMR and ESR) which distinguish energy levels as well as by ORD, gas chromatography and dipole moments which measure other molecular properties.

Major Findings:

Quantum Chemistry of Therapeutics and Therapeutic Interference (with Dr. Greenblatt).

Electron density at the ring nitrogen, the energy of the highest occupied molecular orbital and the energy of the lowest empty molecular orbital of a large series of acridine compounds were calculated by the LCAO method. These were studied in relation to the pKa and therapeutic indices of these compounds. For the Gram positive organisms investigated, the toxicities of the compounds correlate well with the electron density at the ring nitrogen and the energy of the lowest empty molecular orbital. Correlation is not as good for the Gram negative organisms studied.

These correlations have been considered in relation to the possible interaction of the acridines with cell membranes and DNA. From this a hypothesis has been evolved for the therapeutic action of acridines as due to the formation of a DNA-acridine complex in which the acridine acts as an electron acceptor.

There is as yet no adequate mechanistic explanation for therapeutic interference, although several hypotheses have been offered. A classic example of such therapeutic interference has been the inhibition of the action of acridine dyes on trypanosomes by certain triphenyl methane dyes. Physical-chemical investigations (X ray diffraction, NMR, spectrophotometry and fluorescence) show that trypaflavin and parafuchsin or crystal violet form a 1:1 complex. This may be, at least in part, the explanation for the therapeutic interference.

Interference between crystal violet and trypaflavin as evidenced by studies on optical rotatory dispersion on DNA, and poly-l-glutamic acid indicates that the mechanism of interference varies according to the substrate.

Electron Spin Resonance Studies (with Dr. Kon). The metal-complexing abilities of aromatic diazo compounds are well known, but the structure of the complex is uncertain, in that knowledge of the precise attachment of the metal to the azo group is unknown. ESR studies with copper complexes now under way, indicate an attachment of the metal to a definite nitrogen atom, rather than to the azo group as an entity.

Significance to NIAMD Research:

The proper understanding of the behavior of biologically active substances at the molecular level is necessary for the understanding of their actions at the cellular level in order to evaluate their contributions to both health and diseased states.

Proposed Course of Project:

The relative bindings of crystal violet and trypaflavin will be investigated in DNA, poly-l-lysine and other substrates for further information as to therapeutic interferences, and the nature of therapeutic actions. The synthesis of copper complexes of N^{15}-azo compounds will be carried out to obtain information on the structure of such complexes.

Publications:

Kon, Hideo, and Sharpless, Norman E.: Nature of the unpaired electron orbital in some low spin oxygen-Co complexes: Spectroscopy Letters 1: 49-54, Jan. 1968.

Serial No. NIAMD-LPB-21
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Instrumental methods for study of structure and stereochemistry of organic molecules.

Previous Serial Number: NIAMD-LPB-19

Principal Investigator: Ulrich Weiss

Other Investigators: Ulli Eisner
John Michael Edwards

Cooperating Units: Herman Ziffer (NIAMD-LPB-23)
Thomas Rüll, College de France, Paris
M. Legrand, Centre de Recherches Roussel-Uclaf, Romainville, France
Isabella L. Karle, Naval Research Laboratory

Man Years

Total:	1 1/2
Professional:	1 1/2
Other:	0

Project Description:

The objectives of this research are the study of the optical rotatory dispersion and circular dichroism of organic molecules having chromophoric groups other than carbonyl, and the application of the findings to questions of structure and spatial arrangement of these molecules.

Methods Employed:

Optical rotatory dispersion (ORD) down to wave lengths below 200 mμ was measured with the Cary 60 Spectropolarimeter, circular dichroism (CD) with the CD attachment to the Cary 60 of the Section of Chemistry, LM, NHI.

Major Findings:

In further cooperation with Dr. M. Legrand, the CD of a large number of α,β-unsaturated acids, esters, and lactones has been investigated.

Besides the effects in the 250 mμ region, found earlier by us, two new bands at shorter wave lengths are usually present; the signs of the three bands alternate. The available data are at present being examined particularly for connections between the signs of these bands and the stereochemistry of the molecule.

Mostly through the use of instrumental methods, the hitherto uncertain structures of several previously known codeine derivatives have been established. In cooperation with Dr. Rüll, decision between two possible structures of dihydrodesoxycodeine E has been made. Anhydrometathebainol, assumed by L. F. Small (1933) to arise through some skeletal rearrangement, has been found to be the normal, non-rearranged product (U. Eisner). Pentachlorooxycodide, obtained (likewise by Small et al.) from reaction of α-chlorocodide with cold sulfuryl chloride, proved too intractable for chemical transformations to yield any insight into its structure; application of various spectroscopic techniques (IR, n.m.r.) was unrewarding in this case. X ray crystallography (Dr. I. Karle) of an ethyl ether of the compound, obtained by Dr. Eisner, revealed a very complicated and in many respects unexpected structure, which shows that apparently unprecedented reactions are involved in the formation of the substance.

Attempts by J. M. Edwards to prepare hitherto almost inaccessible analogs of the vitamin, biotin, by a simple one-step reaction did not yield the desired compounds thus far, for unknown reasons. Instead, a number of unexpected products were obtained, and their structures elucidated. One of these, identified with a known substance, suggested a one-step approach to a group of substances recently prepared elsewhere by more involved processes and given the general name "propellanes." This class of compounds contains a very rigid ring system; since it is currently assumed that such rigidity favors marked physiological activity, this new approach to the synthesis of propellanes was explored, and diketones derived from [3.3.3] and [4.3.3] propellane were obtained very readily. Another, nitrogen-containing compound was isolated from a reaction of glyoxal with ethylene diamine. It reverts to the starting materials with great ease.

Proposed Course of Project:

The evaluation of the CD data on α,β-unsaturated acids and lactones is to be completed, and the investigation to be extended to other classes of such compounds. Connection between sign of the various CD bands and stereochemical features of the molecules is to be studied further with the help of model compounds of known configuration and rigid molecular geometry.

Publications:

Eisner, U., and Weiss, U.: Derivatives of morphine. V. The structure of anhydrometathebainol. J. Org. Chem. 33: 1264-1265, March 1968.

Weiss, U., Rüll, Th., and Bradley, R. B.: Derivatives of morphine VI. The structure of dihydrodesoxycodeine E, the product of electrolytic reduction of 14-bromocodeinone. J. Org. Chem. In press.

REPORTED IN PRESS - 1967

Ziffer, H., Weiss, U., and Charney, E.: Optical activity of non-planar conjugated dienes. IV. Interacting chromophores in gliotoxin. Tetrahedron 23: 3881-3886, Oct. 1967.

Serial No. NIAMD-LPB-22
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemistry and biosynthesis of natural pigments and other natural compounds

Previous Serial Number: NIAMD-LPB-20

Principal Investigator: Ulrich Weiss

Other Investigators: Robert J. J. Ch. Lousberg, NIAMD, LPB

Cooperating Units: Professor C. A. Salemink and students, University, Utrecht, The Netherlands
Dr. Keith S. Brown, Jr., Centro de Pesquisas de Produtos Naturais, Universidade do Rio de Janeiro, Rio de Janeiro, Brasil

Man Years

Total:	1 1/2
Professional:	1 1/2
Other:	0

Project Description:

Objectives:

Isolation in pure form, and elucidation of the chemical constitution, of the bright red photosensitizing pigments produced by ascomycetous fungi of the genus Elsinoe, and of other natural pigments of possibly related nature.

Methods Employed:

Standard organic synthetic and analytical procedures.

Major Findings:

Additional study by instrumental methods, especially some recently introduced techniques of nuclear magnetic resonance (n.m.r.) spectroscopy, have yielded unequivocal proof for the structure of the mold pigment elsinochrome A; solvent shifts of n.m.r. bands proved instructure, but the clearest evidence came from an investigation of the nuclear Overhauser effect, which constitutes the first application of this new n.m.r. technique to a system consisting of a proton and a methoxy group in adjacent positions on an aromatic ring (Lousberg and Paolillo). This situation is common among natural compounds of medicinal or biochemical interest, so that proof of applicability of the Overhauser effect in such cases should be of considerable methodological value. The stereochemistry of elsinochrome A has been established.

The structure of elsinochrome D, a pigment formed by some species of Elsinoe growing on a modified medium, has been elucidated in part, and that of a related pigment cercosporin has been established. Three strongly fluorescent compounds are present in small amounts in extracts of Elsinoe. One of those, characterized by a yellowish-green fluorescence, has been identified as di-(2-ethyl hexyl)-phthalate, which has also been found in extracts from other organisms, but is probably a contaminant. The UV spectrum of the second compound seems to be that of a phenanthrene; hardly any naturally occuring non-quinonoid phenanthrenes seem to be known. Only trace amounts of the third compound (intense blue fluorescence) have been available thus far.

Because of suspected relationships with the elsinochromes, certain aphid pigments have been studied. The color dimorphism (pink and green) in Macrosiphum liriodendri is due to differences in carotenoid pigments, especially the pink ones being of a type found before only in certain bacteria. The color difference in parthenogenetic individuals of one colony, which is puzzling because of the genetic identity of the individuals, may thus be explicable through differences of the bacterial symbionts rather than of the insects themselves.

The orange pigment neriaphin of Aphis nerii has been investigated in cooperation with Dr. K. S. Brown, isolation and chemical transformations being performed by him, instrumental investigation at NIH. Neriaphin is closely related to previously known aphid pigments which, however, result from coupling of two units of the same type. Neriaphin is thus of considerable biogenetic interest.

Proposed Course of Project:

Investigation of the structure of elsinochrome D and of the two remaining fluorescent trace metabolites of Elsinoe is to be continued. Stereochemistry and possible biosynthetic role of neriaphin are to be examined. Two minor copigments are to be studied. Complete chemical characterization of the unusual carotenoids of Macrosiphum is to be attempted.

Serial No. NIAMD-LPB-23
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:

1. A study of the mechanisms and modes of photochemically induced valence tautomerisms and dimerizations of natural products.
2. A study of the interdependence of the structure and stereochemistry of dienes, α,β-unsaturated ketones and α,β-unsaturated lactones by means of optical rotatory dispersion.

Previous Serial Number: NIAMD-LPB-21

Principal Investigator: Herman Ziffer

Other Investigators: John Williams

Cooperating Units: U. Weiss (NIAMD-LPB-21)
K. Yamaoka
C. H. Robinson, Johns Hopkins Medical School

Man Years

Total: 1 1/4
Professional: 1 1/4
Other: 0

Project Description:

Objectives:

1. To learn more concerning the nature and reactivity of the excited state of α,β-unsaturated ketones.
2. To examine the influence of structural changes on the optical rotatory dispersion of dienes, α,β-unsaturated ketones and α,β-unsaturated lactones.

Methods Employed:

The Cary 60 Spectropolarimeter has been used to obtain ORD measurements. Standard organic chemical techniques were employed for the synthesis and proof of structure of a number of compounds. Along with the usual spectroscopic measurements (NMR, IR and UV) we have used the Varian time averaging computer in conjunction with our NMR equipment to obtain several important NMR measurements necessary for structure determinations.

Major Findings:

1. [illegible] activity of Actinomycin D (AMD) as well as that of the ring-opened monolactone monocarboxylic acid and dicarboxylic acid have been determined in several solvents. The optical activity associated with the long wavelength band (450-440 mμ) of AMD is weak, however, a strong optically active band is present at ∿ 380 mμ. This band has been shown to require the presence of both lactone rings and several explanations of this effect were proposed. On complexing AMD with DNA the long wavelength absorption band of AMD becomes strongly optically active. From quantitative studies of the ORD curves at different phosphate residue-to-AMD (P/A) ratios it was shown that the shape and magnitude of the curve at a P/A ratio of 6 could be calculated from the data obtained at a P/A ratio of 15. There were no qualitative differences between the complexes of AMD with native or denatured DNA. In order for DNA to complex with AMD both lactone rings of the AMD are required.
2. The structures of several photodimers were studied and the structures either partially or completely elucidated.
3. A comparison of the ORD and CD curves of several α,β-unsaturated ketones with structurally related dienes showed that, although the theoretical treatments of the optical activity associated with the $\pi \rightarrow \pi^*$ transition of these compounds are very similar, some of the curves differ significantly. In addition the CD curves of several α,β-unsaturated ketones show the existence of a strongly optically active transition that was unknown previously.

Significance to NIAMD research:

Optical rotatory dispersion curves are commonly used in assigning structures and stereochemistry to biologically important molecules. The continuing study of the ORD of dienes, α,β-unsaturated ketones and other compounds is an attempt to explore the areas and situations where these assignments can be used reliably.

The increased knowledge derived from studies of the mode and mechanism of photochemical reactions permit the synthesis of new and more reactive compounds that are either very difficult or impossible to prepare by more conventional methods.

Proposed Course of Project:

1. To continue the study of the photochemical rearrangement of β,γ-unsaturated ketones in order to determine the scope and learn more of the mechanisms of this reaction.
2. To continue the study of the photodimerization of α,β-unsaturated ketones as well as a number of quinones in order to learn more concerning the stereochemistry of the reaction.
3. To continue the study of the relationships, between ORD (as well as CD) of dienes, α,β-unsaturated ketones, and α,β-unsaturated lactones with structure and stereochemistry.

Publications:

Williams, J. R., and Ziffer, Herman: The photochemical synthesis of A-Nor-5α,10α-steroids. Chem Comm. 1967: 469, May 1967.

Ziffer, H., and Williams, J. R.: Citraconic anhydride photodimer. Structure and stereochemistry. J. Org. Chem. 33: 920-921, Feb. 1968.

Ziffer, H., Yamaoka, K., and Mauger, A. B.: Optical properties of Actinomycin D. I. Influence of the lactone rings on its optical activity. Biochem. 7: 996-1001, March 1968.

Yamaoka, K., and Ziffer, H.: The optical properties of Actinomycin D. II. Optical activity of the DNA complex. Biochem. 7: 1001-1008, March 1968.

Ziffer, H., and Robinson, C. H.: On the optical activity of α,β-unsaturated ketones. Tetrahedron. In press.

Serial No. NIAMD-LPB - 24
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of altitude (hypoxia), exercise, and other environmental stresses on physiological, biochemical and pathological mechanisms in animals.

Previous Serial Number: LPB-22

Principal Investigator: Paul D. Altland

Other Investigator: Mr. Howard Brubach

Cooperating Units: Dr. Benjamin Highman, Section on Pathologic Anatomy, Laboratory of Experimental Pathology, NIAMD, LEP-2 A.

Dr. John W. Adamson, Hematology Section, Clinical Pathology Department, Clinical Center

Man Years (July 1967 through June 1968)

Total: 4½
Professional: 1½
Other: 3

Project Description:

Objectives:

To determine the physiological and biochemical changes that occur during exposure to altitude, exercise, and cold, and to study the mechanism of action of cross adaptation between these stresses. Emphasis is placed upon serum enzyme and tissue changes.

To determine physiological and biochemical changes that occur in animals exercised in an atmosphere containing various O_2 concentrations in inert gases such as SF_6, A, N_2, and He, and to determine the influence of particulate matter, such as smoke, upon these changes.

To develop a better standardized and easily applied erythropoitin bioassay and its implementation in the evaluation of various anemic and polycythemic states (Drs. Adamson & Altland).

Methods Employed:

Biochemical tests for enzymes and isoenzymes. Clinical and histopathological tests. Two altitude chambers, a gas chamber for small animals equipped with arm inserts for manipulation, small animal treadmill, wheel type exerciser, designed to permit study of rats exposed to various gas mixtures and standard x-irradiation equipment.

Major Findings:

Exercise of untrained rats combined with exposure to 21,000 ft. (4 hr/d) produced elevations in serum aldolase, glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and lactic and malic dehydrogenases, and a reduction in alkaline phosphatase. In addition, exercise induced an increase in incidence of all five isoenzyme bands of lactic dehydrogenase and a prominent mitochondrial band of malic dehydrogenase. There was an increase in the incidence of fatty changes in the heart and muscle after 1 day of exercise-altitude exposure and a high incidence of fatty changes in the liver from 20 hrs to 7 days. Exercise training at ground level (6 hr/day) for 21 days lowered or prevented enzyme changes and reduced the fatty changes in the thigh muscle and liver produced after 1 to 3 periods of exercise at altitude. In earlier studies, exercise training failed to prevent fatty changes and other lesions following a period of severe altitude hypoxia (4 hours at 32,000 ft.).

In rats which were given dimethyl sulfoxide and required to exercise at ground level for 12 hours, the oxidative and glycolytic enzymes in the serum increase 2 to 4 times above rats exercised without the drug, and fatty changes in the tissues were increased. In trained rats exercised 12 hours and pretreated with dimethyl sulfoxide the incidence of fatty changes increased in the liver and abnormal serum enzyme values persisted.

In studies with rats exposed to low O_2 concentrations mixed with inert gases such as SF_6, A, N_2, and He, the hypoxic tolerance was highest in He-4.9% O_2 and lowest in A-4.9% O_2 and SF_6-4.9% O_2.

The advantage of He was found to be due to the higher arterial O_2 saturation (50.6% compared with 42.3% for N_2-4.9% O_2) and the lower mean body temperature (29.7° compared with 32.4°C in N_2-4.9% O_2), and low density with its favorable influence on turbulence and diffusion.

Significance to NIAMD Research:

The beneficial effects of ground level training such as the reduction of mortality, the reduction of leakage of enzymes into the serum, and the prevention of fatty changes in striated muscle and liver in rats exercised at high altitude were shown. It is indicated that exercise training protects the permeability of cellular membranes, and thus helps maintain the integrity of the cell. A probable outcome is the absence of pathological change in the cells of the trained rats. These results provide basic data on the influence of the adaptation of one stress (exercise) on the response to another stress (hypoxia-exercise at altitude). Some insight is obtained into the biochemical basis for the statement that trained men perform better than untrained men at altitude.

The work with inert gases emphasized the value of the use of a gas with a low density to increase the arterial O_2 saturation of rats in an hypoxic atmosphere.

Proposed Course of Project:

Experiments will be conducted to evaluate the biochemical, physiological, and pathological changes that occur in animals subjected to multiple stresses and to evaluate the effects of cross adaptation between stresses. Special attention will be given to the influence of exercise and smoke inhalation in rats subjected to various hypoxic gas mixtures.

Publications:

Garbus, Joel, Highman, B. and Altland, P. D.: Alterations in serum enzymes and isoenzymes in various species induced by epinephrine. Comp. Biochem. Physiol. 22: 507-516, 1967.

Highman, B. and Altland, P. D.: Effect of dimethyl sulfoxide, exercise, and training on rat serum enzymes and tissues. Am. J. Physiol. 213: 779-782, Sept. 1967.

Nelson, B. D., Highman, B., and Altland, P. D.: Oxidative phosphorylation during acclimation in rats. Am. J. Physiol. 213: 1414-1418, Dec. 1967.

Altland, P. D., Highman, B., Nelson, B. D.: Serum enzyme and tissue changes in rats exercised repeatedly at altitude: Effects of training. Am. J. Physiol. 214: 28-32, Jan. 1968.

Altland, P. D. and Highman, B.: Sex organ changes and breeding performance of male rats exposed to altitude: Effect of exercise and physical training. J. Reprod. Fertility (in press)

Altland, P. D., Brubach, H. F., and Parker, M. G.: Effects of inert gases on tolerance of rats to hypoxia. J. Appl. Physiol. (in press).

Serial No. NIAMD-LPB- 25
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of environmental stresses on cellular homeostasis.

Previous Serial Number: None

Principal Investigator: Michael P. Dieter

Other Investigators: None

Cooperating Unit: None

Man Years (July 1967 through June 1968)

Total:	1½
Professional:	1
Other:	½

Project Description:

Objectives:

To make a comparative study of the biochemical mechanisms responsible for the control of cellular homeostasis in lymphatic tissues.

Methods Employed:

Spectrophotometric assay of key metabolic enzymes, colorimetric determination of tissue concentrations of reduced, oxidized, and total vitamin C.

Major Findings:

Studies have been initiated in stress or stress-adapted animals to

1. Determine the metabolic nature of the lympholytic response,
2. Further define the hormonal control of cellular homeostasis, and
3. Ascertain the extent of vitamin C-hormone interrelationships in lymphatic tissues.

Acute exercise stress (10 hours) of untrained or physically trained rats decreased the growth rate of thymus and spleen and significantly altered tissue enzyme activity. Physical training did not prevent a decrease in glycolytic enzyme activity (lactic dehydrogenase) caused by acute exercise, but did cause a decline of direct glucose oxidation via hexose monophosphate shunt, and increase the availability of three carbon intermediates by augmenting glutamic-pyruvic transaminase activity. Adrenal hypertrophy and vitamin C depletion also occurred in the exercised rats, implicating adrenocortical hormones in the lymphatic tissue responses; the change in the enzymatic pattern suggested that there was a rapid and coordinated regulation of glycolytic activity correlated with the severity of stress and/or increase in adrenal activity.

Discontinuous altitude exposure (25,000 ft. simulated altitude, 5 hr/day for 21 days) decreased thymus weight and increased spleen weight. Thymus lactic dehydrogenase activity was increased and spleen glucose-6-phosphate dehydrogenase and glutamic-oxalacetic transaminase activity decreased. Five hours altitude exposure (25,000 ft. simulated altitude) of unacclimated rats decreased thymus and spleen weights, and altered tissue enzyme activity. Lactic dehydrogenase activity was increased in thymus and spleen. In addition there was an increase in the activity of spleen glycerol-phosphate dehydrogenase, glutamic-pyruvic transaminase, and glutamic-oxalacetic transaminase. Adrenal hypertrophy and vitamin C depletion occurred in the rats exposed to altitude stress.

The concentration of total vitamin C in the thymus and spleen of exercised rats was elevated above that of unexercised rats; there was no change in the concentration of ascorbic acid, but a two-fold increase in the concentration of dehydroascorbic acid occurred. The ratio of ascorbate/dehydroascorbate in unexercised rats (3-8) thus fell to about unity in lymphatic tissues of exercised rats. Exercise stress did not affect hepatic total vitamin C or ascorbic acid concentration, but decreased the concentration of dehydroascorbic acid; the ratio of ascorbate/dehydroascorbate in liver rose from a value of close to unity in unexercised rats to as high as six in liver of exercised rats. These preliminary results illustrate the changes that occur in the concentration and oxidation state of lymphatic or hepatic vitamin C of stressed rats. The relationship between hepatic ascorbic acid synthesis, vitamin C transport, and possible vitamin C utilization by the lymphatic tissues are being considered.

Significance to NIAMD research:

The findings indicate that stress alters key metabolic enzymes directing the metabolism of glucose in lymphatic tissues, curtailing monophosphate shunt biosynthetic activity and favoring glycolytic activity. These mechanisms may better enable the lymphatic system to withstand stress exposure during the early, critical period of tissue development.

Serial No. NIAMD-LPB- 26

1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The physicochemical properties of biological material and related chemicals at interfaces.

Previous Serial Number: NIAMD-LPB-23

Principal Investigator: N. L. Gershfeld

Other Investigators: Dr. Mitsuo Muramatsu

Cooperating Units: Dr. C. S. Patlak, NIMH:B

Man Years (July 1967 through June 1968)

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

(a) To measure the intermolecular cohesive and repulsive energies in condensed monomolecular films.

(b) To study the influence of film structure on reactions occurring in monomolecular films.

(c) To study the properties of cell surfaces within the framework of the findings of (a) and (b).

Methods Employed:

1. The rates of film desorption at constant surface pressure are measured from which the chemical potential of the monolayers in various states or aggregation are determined.

2. Free energies of binding to surface components for steroid hormones are determined by using C^{14} labeled steroids.

3. Surface viscosity of monolayers measured with a torsion pendulum device.

Major Findings:

1. The thermodynamic properties of the hydrocarbon region of a condensed monolayer have been evaluated. The following values are for the process involved in the formation of the liquid-condensed film (which is equivalent to the liquid-crystalline phase in bulk systems). For an 18 carbon compound at 25°: ΔF_c = -5.8 K cal/mole, ΔE_c = -23 K cal/mole, and ΔS_c = -58 e.u./mole. The value for ΔE_c is intermediate between that for a hydrocarbon liquid and solid.

2. It has been demonstrated that the steroid hormones androsterone, aldosterone, and desoxycorticosterone at very low concentrations (10^{-7}M) can

   (a) alter the structure of monomolecular films and

   (b) increase the rate of polymer formation in films by factors of 30-40 fold.

Significance to NIAMD Research:

The large value for ΔE_c (-23 K cal/mole) is direct evidence that the interaction between oriented lipid molecules makes a large contribution to the stabilization energy in structures of which they are part. This would be particularly true for the lipids in cytoplasmic membranes.

Demonstration of steroid-monolayer interactions at low steroid concentrations is an encouraging indication that the monolayer system will be a useful tool for studying the structure-function relations of steroid hormones.

Proposed Course of Project:

The studies on monolayer interactions will be continued to evaluate the stabilization energies in the polar region of the monolayer. The influence of chemical structure on the rate of reactions at the surface will be examined. Development of techniques for studying the properties of cell surfaces will also be emphasized.

Publications:

Patlak, C. S. and Gershfeld, N. L.: A theoretical treatment for the kinetics of monolayer desorption from interfaces. J. Colloid and Interface Sci. 25: 503-513, Dec. 1967.

Gershfeld, N. L.: Cohesive forces in monomolecular films at an air/water interface. Publication in "Molecular Aggregation in Biological and Related Systems", Advances in Chemistry Series, of the American Chemical Society. (in press)

REPORTED IN PRESS - 1966-1967

Pak, C.Y.C. and Gershfeld, N. L.: Steroid Hormones and Monolayers. Nature 214: 888-889, May 1967.

Gershfeld, N. L. and Good, R. J.: Line tension and the penetration of a cell membrane by an oil drop. J. Theoret. Biol. 17: 246-251 Nov. 1967.

Serial No. NIAMD-LPB- 27
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of Metabolic Activity in Microorganisms.

Previous Serial Number: LPB-24

Principal Investigator: Ellis S. Kempner

Other Investigator: Jay H. Miller

Cooperating Units: none

Man Years (July 1967 through June 1968)

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

An understanding of the control of metabolic activity in unicellular organisms.

Methods Employed:

Culture of microorganisms in the presence of radioactive or other compounds: chemical and physical fractionation of cellular materials by ultracentrifugation, chromatographic separation, and chemical extraction.

Major Findings:

Studies of light production by Photobacterium fischeri reported in the previous annual report were concluded. It was found that the growth medium, nutrient broth, contained an inhibitor specific for bioluminescence. The inhibitor acts by binding directly to the luciferase molecules present in the cell, and is without effect on the synthesis of these molecules.

Studies of the protozoan, Euglena gracilis, have been continued. Studies of the localization of enzymes and other macromolecules in cells stratified by centrifugation have been extended. Various attempts to dislodge enzymatic activity from particulates within the living cell were not successful, suggesting that a viable organism is capable of maintaining a highly organized metabolic apparatus against external forces. The ability of cells to recover from centrifugation-caused stratification was determined by colony formation and oxygen utilization.

Isolation of presumed peptide material from exponentially-growing Euglena cells was begun. The quantity of material isolated represented 4 to 8% of the cellular carbon and appears to contain 50% arginine. The detailed structure and metabolic significance of these peptides is being examined.

Significance to NIAMD Research:

Researches on Euglena gracilis have enabled the development of a system for studying differentiated cellular structure with the technical ease usual with bacteria. Using this system, new insight into the structure and function of cells is being gained.

Proposed Course of Project:

1. To study the metabolic relationships of synthetic processes in Euglena in relation to the highly organized cellular structure which has been elucidated.

2. To extract, separate, and identify the peptide materials in Euglena and to establish their role in cellular metabolism.

Honors, Awards, Etc.

Invited to act as advisor on microbiology at the Second Surtsey Research Conference held at Reykjavik, Iceland, June 1967.

Publications:

Kempner, Ellis S.: Trace metal analysis of nutrient broth. Appl. Microbiol. 15: 1525-1526, Nov. 1967.

Kempner, E. S. and Hanson, F. E.: Aspects of light production by Photobacterium fischeri. J. Bacteriol. 95: 975-979, Mar. 1968.

Kempner, E. S. and Miller, Jay H.: The molecular biology of *Euglena gracilis.* IV. Cellular stratification by centrifuging. Exptl. Cell Research (in press)

Kempner, E. S. and Miller, Jay H.: The molecular biology of *Euglena gracilis.* V. Enzyme Localization. Exptl. Cell Research (in press)

Serial No. NIAMD-LPB- 28

1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigation of the macromolecular organization of living matter.

Previous Serial Number: LPB-25

Principal Investigator: L. W. Labaw

Other Investigators: none

Cooperating Units: none

Man Years (July 1967 through June 1968)

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

To gain information about the macromolecules that are essential constituents of living matter and to see how they are arranged in the structures they form. To study certain of these macromolecules, such as viruses and other proteins, in purified form after isolation from the living material. To improve the resolution of the electron microscope and to interpret the way in which images are formed near its limit of resolution.

Methods Employed:

The electron microscopy of microorganisms, cells and tissues in suspension or thinly sectioned. The physicochemical characterization of macromolecular components isolated from such material using electron microscopy, x-ray diffraction, and similar established techniques.

Major Findings:

The assumption that bovine-liver catalase molecules pack as spheres, when this protein is crystallized, gave a good explanation of the observed periodicities on shadowed crystal replicas in the electron microscope. The packing arrangement proposed from electron microscopic data fitted the P3, 21 space-group determined by Prof. M. G. Rossmann, Purdue University, from x-ray analysis of these crystals, but the x-ray unit cell lengths indicated either that the molecules were not quite in the proposed positions, or they were not strictly spherical. Spherical molecules in the proposed positions also did not give a simple explanation for the enhanced intensity of x-ray reflections in a Star of David design which is characteristic of 9° precession photographs when the x-ray beam parallels the unique crystal axis. A third difficulty was the failure to predict rows of holes seen on electron micrographs of embedded-crystal sections cut perpendicular to the two-fold axis. All of these discrepancies disappear if one assumes the catalase molecule contains four equal or nearly equal subunits arranged at the corners of a tetrahedron. Such a tetrahedral molecule packed in the originally proposed positions according to P3, 21 symmetry not only explains most of the enhanced reflections in the diffraction photographs and the rows of holes looking along the two-fold axis, but gives an approximately spherical subunit diameter, as calculated from the unit cell dimensions, of 50.0 ± 1 Å and 53.5 ± 1 Å compared with a diameter of 52.2 Å, calculated from 1/4 the molecular weight of 250,000 and a density of 1.4 gms/cm^3.

While observing bovine thyroid polyribosomes for Dr. J. E. Rall and Dr. J. Robbins, a collagen-related fibrous structure was observed which had the appearance of 200 Å diameter spherical particles spaced 600 to 700 Å apart along a thin fiber. In very clean preparations, these thin fibers appeared as bundles of smaller fibers, similar to tropocollagen molecules. These strands were not collagen since they were unaffected by collagenase.

Significance to NIAMD Research:

The use of the electron microscope to determine the crystal structure of protein crystals is particularly important when applied to crystals which cannot be grown large enough for a more complete structure study by x-rays.

With increased emphasis on fine structure in electron microscopy, it is pertinent to examine the mechanism of image formation to define the conditions under which one can be assured that observed periodicities on images in the electron microscope have a direct spatial relationship with the object being examined when one is operating near the resolution limit of the instrument.

Proposed Course of Project:

To investigate other protein crystals in the electron microscope using replica, negative staining, and direct transmission techniques to determine their structures.

Publications:

Labaw, L. W.: The crystal packing of Thyroglobulin. Proc. Electron Microscope Soc. of Am., Chicago, Ill., Aug. 29-Sept. 1, 1967, Pub. by Claitor's Book Store, Baton Rouge, La., pp. 194-195, Sept. 1967, Ed.-Claude J. Arceneaux.

Rossmann, M. G. and Labaw, L. W.: A comparison of electron microscopy and x-ray diffraction results for Ox liver catalase crystals. J. Mol. Biol. 29: 315-316, Nov. 1967.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOPHYSICAL CHEMISTRY

The main objectives of this laboratory originated when, about 20 years ago, A. Szent-Györgyi and some of his co-workers switched their location from Budapest to Bethesda and joined up with a group interested in the chemistry of proteins. As a consequence, the main objectives of this laboratory became the study of the muscle proteins involved in muscular contraction and the plasma proteins involved in the clot formation with the idea that a thorough study of these proteins and enzymes will lead to the understanding of their biological role.

The work of this laboratory may be treated under three headings: elucidation of the events taking place when muscle converts chemical energy into mechanical work; the study of protein-protein interaction exemplified when fibrinogen clots; and the investigation of the structure of the above mentioned and other biologically important macromolecules when these are in solution.

In the past years, we have had the occasion to report significant contributions to the above objectives. This year we can report some further progress.

The effects of salts of monovalent ions on ATP-induced isotonic shortening of glycerol-treated muscle fibers seems to be one of ionic strength rather than of ionic species; but the effects of salts of divalent ions vary according to the species of cation. (Hotta, Bowen).

Skeletal muscle contains transamidase. The activity is very low; it amounts to only a few percent of the activity of liver. In connection with this finding, we observed that the stabilizing enzyme from guinea pig liver catalyzed the incorporation of amines and connected myosin molecules (in a manner analogous to the cross-linking of fibrin molecules) into higher aggregates. These and other observations suggest that this enzyme may have a role in muscular contraction. (Laki, Benkö).

Muscle is an energy transducer in which the chemical energy of ATP is converted to work. It is apparent that in the primary transducer the decomposition of ATP must be coupled to mechanical events.

F-actin, which normally contains firmly bound ADP, is known to behave as an ATPase under the influence of ultrasonic vibration. Our investigations indicate that N-ethyl maleimide (NEM), which under normal conditions reacts with only two sulfhydryls in actin, will react with at least one additional SH group when the nucleotide is released by ultrasonic vibration. In addition, we find that ATP, but not ADP, is a powerful inhibitor of NEM incorporation during sonication. Actin continues to react slowly with NEM after sonication, but addition of ATP will stop this reaction. Only the NEM which is incorporated into actin during sonication has an inhibiting effect on its ATPase activity.

These experiments indicate that in the "active center" of actin, a cysteine residue reacts with ATP in such a manner that phosphate is released and the rest of the ATP molecules "acylates" the enzyme. In the final step, mechanical deformation promotes the acyl-intermediate (adenosine-diphosphoryl-actin) to release the nucleotide as ADP. (Alving, Laki).

Removal of C-terminal phenylalanine from actin does not affect the latter's ability to combine with myosin. It does, however, influence the rate and extent of the polymerization of actin. (Field, Bowen).

We have a continuing interest in the elucidation of the clotting of fibrinogen. The alignment of the fibrin molecules in the clot is still debated. Based on the available data, a model of the clot was constructed. This model exhibits a periodicity reminding one of the pattern seen in the self-assembly of the collagen molecules. This similarity of pattern may be a partial explanation for the clot serving as a matrix for fibroblasts in wound healing. (Thomas).

When fibrinogen clots, two negatively charged peptides are liberated and the altered fibrinogen molecules aggregate to a network structure.

The physiological effect of intravenous injection of bovine peptide-B is a prolonged rhythmic vasoconstriction and is unlike any previously described vasopressor peptide. Chemical modification of this peptide has produced an active vasopressor portion of the intact peptide. The human peptide, which contains a phosphate group covalently bound to a serine residue, has also been demonstrated to be an active vasopressor.

In the normal clotting process, an enzyme cross-links the fibrin molecules in the clot. We have found previously that not only plasma, but also tissues, contain clot stabilizing enzymes. Since the stabilized clot is involved in wound healing, we explored whether the clot stabilizing enzyme has a role in other functions.

There are reports in the literature indicating that fibrin clot formation is involved in the development of atherosclerotic plaques. Since cell proliferation appears to be connected with the development of the plaques, we undertook a study to investigate whether the clot stabilizing enzyme is implicated in atherosclerosis. Rabbits were kept on high cholesterol diets and after 8 weeks, the clot stabilizing enzyme content of their aorta was compared to normal animals. A threefold increase in enzyme content was found in the aorta of the atherosclerotic animals. Also, the diseased portion of the aorta exhibited a threefold increase as compared to the non-diseased portion. (Benkő, Laki).

Bovine fibrinogen has been modified by dithiothreitol (Cleland's Reagent) at pH 9 and 10 at various ratios of reagent to total fibrinogen disulfide content. The results have been examined by the addition of thrombin and by the ultracentrifuge. Of great interest is the observation that at pH 9.0 the ability of the modified fibrinogen to clot upon the addition of thrombin slowly returns. This is indicative of the rupture of a very small number of disulfide bonds that will affect clotting ability, followed by the gradual return to the conformation which allows clotting upon thrombin action. (Gladner, Murtaugh).

The subcutaneous injection of anti-inflammatory drugs into rats causes the appearance of proteolytic activity in the subcutaneous compartment of rat skin which can be extracted into NaCl. Experimentation now shows the presence of more than one protease in the saline extracts. One of these is a protease which has a limited chymase activity capable of converting fibrinogen to fibrin by releasing a single large peptide. A new type of carboxypeptidase has also been observed in these skin extracts. It possesses the capability of removing a C-terminal phenylalanine when a proline is the penultimate amino acid residue. Unlike the classical carboxypeptidases, it is inhibited by DFP. (Gladner, Houck).

When fibrinogen is digested by plasmin, some of the early digestion products are very similar to fibrinogen, but can be distinguished by their reactivity to thrombin and by their detailed hydrodynamic behavior. Diffusion studies have confirmed the essential homogeneity of the isolated products and enabled the calculation of molecular weights, showing a progressive, yet quite specific, conversion of fibrinogen to fragments having varying biological activity and lower and lower molecular weights. (Carroll, Marder).

Sedimentation and diffusion studies for determination of heterogeneity and molecular weight, under various conditions of preparation and chemical treatment, were carried out on yeast aldehyde dehydrogenase, maleate isomerase, epoxysuccinate hydratase, and others. (Jakoby, Carroll).

Biochemical studies on unusual amino acids and metabolites in living systems led to the isolation and identification of S-sulfo-L-cysteine from the urine of a mentally retarded child with severe brain damage and dislocated lenses which resulted in the discovery of a new disease, sulfite oxidase deficiency. Continued studies were made on this interesting abnormality in man. Since a patient with sulfite oxidase deficiency lacks inorganic sulfate, and since the latter is the ultimate precursor of the organic sulfate esters in such materials as myelin, cartilage and acid mucopolysaccharides, it is a possibility that this deficiency would be reflected in the tissues which, in turn, might contribute to the development of the pathological changes in the patient. To evaluate this hypothesis, studies were undertaken towards 3 parameters: sulfatides or sphingoglycolipids, tyrosine-O-sulfate, and indoxyl-3-sulfate. (Irreverre and Mudd, et al.).

The fundamental studies of polystyrenesulfonic acid as a model polyelectrolyte are continuing and show that large changes in dimensions take place as functions of temperature as well as ionic strength and even particular species of ion. Such effects indicate a specificity related to size and chemistry of individual ions, and point to their particular influences on the confirmation of the polymer. (Carroll, Eisenberg).

In collaboration with the Laboratory of Metabolism of the National Heart Institute, a theoretical analysis was made of data on the distribution of fatty acids between aqueous and non-aqueous phases, and on the binding of fatty acid anions to serum albumin. From competition studies, it was shown that the sites of strong binding for chloride are not identical with those for strong binding of fatty acid anions. (Simpson, Spector).

A glass electrode for accurate evaluation of sodium ion activity has been constructed for study of both the properties of the glass membrane and for the binding of sodium ions to model compounds, polymers and proteins. Measurements with this electrode have reproduced results already found in this laboratory on the binding of sodium ions to malonic acid. (Dygert, Saroff).

Serial No. NIAMD-LBC 1
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Proteins, enzymes and peptides involved in blood coagulation and inflammation.

Previous Serial Number: LBC-7

Principal Investigator: Dr. Jules A. Gladner

Other Investigator: Mrs. P. A. Murtaugh

Cooperating Units: Dr. John C. Houck, Children's Hospital, Wash., DC and the George Washington University Medical School, Wash., D.C.
Prof. K. Hyashi and Prof. T. Suzuki, Institute for Protein Research, Osaka University, Osaka, Japan

Man Years
Total: 2
Professional: 2

Project Description:

Objectives: The thrombin catalyzed conversion of fibrinogen to fibrin is an excellent system to follow the details of the reactions of "limited proteolysis". This reaction is vital to life processes. In addition to this, related processes such as the biochemistry of the inflammatory response are concurrently under investigation.

Methods: Methods used were those of peptide and protein-enzyme fractionation, purification, and characterization, and protein modification. All methods of physical-biochemistry were employed where needed. Physiological techniques were utilized to examine the biological activity of various peptides. Where applicable, new techniques were developed.

Major Findings: Modification of Fibrinogen: Bovine fibrinogen has been modified by dithiothreitol (Cleland's Reagent) at pH 9 and 10 at various ratios of reagent to total fibrinogen disulfide content. The results have been examined by (1) the addition of thrombin, and (2) ultracentrifugally. When the ratio of reagent to disulfide is low, no difference can be observed between the modified and native fibrinogen when viewed in the ultracentrifuge. When the ratio is increased beyond

10:1 (total reagent to total fibrinogen disulfide), the modified fibrinogen shows at least two components. At very high ratios, there are more than two components. At all ratios beyond one to one, the fibrinogen no longer clots upon the addition of thrombin. However, and of great interest, is the observation that at pH 9.0 and a ratio of one to one, the ability of the modified fibrinogen to clot upon the addition of thrombin slowly returns. This is indicative of the rupture of a very small number of disulfide bonds that will affect clotting ability, followed by the gradual return to the original conformation or a conformation which allows clotting upon thrombin action. At present it is not known whether or not this is due to failure of thrombin to act on the modified fibrinogen, or if clotting does not ensue following thrombin action on the modified fibrinogen. It is planned to thoroughly investigate all the parameters of this reaction since the aggregation (clotting) of the fibrin is the "key reaction" of the entire blood coagulation mechanism. We then will view the reaction from the standpoint of molecular biology.

The Induction of Proteases: The subcutaneous injection of anti-inflammatory drugs such as cortisone, indomethacin, and oxyphenybutazone into rats causes the appearance of proteolytic activity in the subcutaneous compartment of rat skin which can be extracted into 0.15M NaCl. The activity is optimal at pH 7.5. That this is actual induction of enzyme(s) is strengthened by the observation that the appearance of this enzymatic activity is blocked when such agents as puromycin, cyclohexamide and actinomycin-D are injected with the inducer anti-inflammatory drugs. These latter agents are known blockers of protein biosynthesis.

Experimentation now shows the presence of more than one protease in the saline extracts. These are as follows:

(1) A protease having a limited chymase activity capable of converting fibrinogen to fibrin by release of a single large peptide. No thrombin activity can be detected in these preparations. The peptide released by this enzyme is under investigation. (Part of this has been reported previously).

(2) The presence of a protease which rapidly inactivates bradykinin. Bradykinin, a nonapeptide, has strong smooth muscle contracting properties, is a powerful pain producer, and is present in many stress conditions such as inflammation, infection, burns, etc. The induction of an enzyme to inactivate bradykinin is a very interesting concept. The enzyme appears to be a "limited protease" in that it has no affect upon bradykininogen the protein precursor of bradykinin. It also shows no affect upon a large peptide obtained chemically from bradykininogen which contains within it the bradykinin structure.

(3) Along with (2) above, a new type of carboxypeptidase has been observed. It possesses the capability of removing a C-terminal phenylalanine when a proline is the penultimate amino acid residue. Unlike the classical carboxypeptidases, it is inhibited by DFP. A complete

study of the specificity of this enzyme is in progress at present.

(Numbers (2) and (3) were carried out in part during the principal investigator's tenure as Visiting Professor at the Institute for Protein Research, Osaka University, Osaka, Japan, during the late summer and fall, 1967).

Similar enzyme induction experiments utilizing tissue culture, human fiberblasts, are beginning to yield results which were found in our studies with rats.

Significance to biomedical research and the program of the Institute: These studies clearly indicate that biological systems are not static but are related one to the other. Thus, during the inflammatory response, an enzyme(s) may be induced which can regulate blood coagulation, release biologically active peptides, and control the activity of other biologically active peptides in the circulation.

Proposed course of project: These studies relating coagulation to other systems will be actively pursued, especially as to the role of hormones and enzyme induction. Whole animal work will also be employed to assay physiological activity of the peptides released from fibrinogen by thrombin. Isolation of these induced enzymes and the products of their action on fibrinogens is proposed. The induction studies will also be carried out with tissue culture as a better source for enzyme isolation.

Publications:

1. Houck, J. C., Patel, Y. M. and Gladner, J. A.: The effects of anti-inflammatory drugs upon the chemistry and enzymology of rat skin. Biochem. Pharmacol. 16: 1099-1111, Aug. 1967.

2. Gladner, J. A.: The action of thrombin on fibrinogen. In Laki, K. (Ed.): Fibrinogen. New York and London, Marcel Dekker, 1968, pp. 87-114.

3. Gladner, J. A., Murtaugh, P.A. and Houck, J. C.: The biological properties of peptides derived from fibrinogen. Biochem. Pharmacol. Special supplement, pp. 259-268, Mar. 1968.

Serial No. NIAMD-LBC 2
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Action of Thrombin on Fibrinogen

Previous Serial No.: NIAMD-LBC 10

Principal Investigator: Dr. A. J. Osbahr

Technical Assistance: Mr. Clarence Israel

Cooperating Unit: Dr. J. Clements, Cardiovascular Research Institute
San Francisco, California

Man Years
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: The chemical structure and physiological role of the peptides released from fibrinogen was investigated.

Methods: The methods applied to study these systems were those of structure determination and sequence analysis with the special application of techniques to evaluate homogeneity and purity of the systems under investigation. More specialized techniques of physiology were utilized when needed to study the biological role of the products of blood coagulation.

Major Findings:

Vasopressor Effect of Peptides Released from Fibrinogen by Thrombin

The physiological effect of intravenous injection of bovine peptide-B is a prolonged rhythmic vasoconstriction and is unlike any previously described vasopressor peptide.

Chemical modification of this peptide has produced an active vasopressor portion of the intact peptide. The possible role of bovine peptide-B is postulated as assisting in local hemostasis by vasoconstriction. The human peptide, which contains a phosphate group covalently bound to a serine residue, has also been demonstrated to be an active vasopressor.

Effect of Thrombin-Induced Peptides from Fibrinogen on the Pulmonary Circulation

Bovine peptide-B and human peptide-AP both increased pulmonary arterial blood pressure 100-250% which was sustained at its elevated level for as long as two hours after a single injection. Modification of these peptides has produced a smaller active fragment and further modification is in progress.

Effect of Thrombin-Induced Peptides from Fibrinogen on the Myosin-B Superprecipitation and Hydrolysis of ATP

Isolated purified myosin B, when pretreated with thrombin-induced peptide from fibrinogen, shows a 50% increase in ATPase activity and a 300% increase in superprecipitation over and above control levels. The peptide is active at a concentration of 10^{-8}M. We suggest that the effect produced here might be a result of the interaction of the highly charged peptide with the myosin B complex. This may enable increased binding of ATP to the actin-myosin complex and thereby result in an increased enzymatic activity. These results may indeed give us an insight into the mechanism involved in the vasoconstriction induced by the peptides.

Significance to biomedical research and the program of the Institute: Out studies indicate that other interesting relationships appear such as the effect of capillary pressure and modification of myosin-actin interaction.

Proposed course of project: Chemical modification and evaluation of physiological role of peptides from fibrinogen will be investigated and tested for their effect in other biological systems.

Publications:

1. Osbahr, A. J., Morris, R. E. and Colman, R. W.: New vasoconstrictor, bovine peptide B, released during blood coagulation. Nature 215, 292-293, Jul. 1967.

2. Bayley, T., Clements, J. A. and Osbahr, A. J.: Pulmonary and circulatory effects of fibrinopeptides. Circ. Res. XXI, 469-489, Oct. 1967.

Serial No. NIAMD LBC- 3
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The structure and properties of synthetic polyelectrolytes as models for charged proteins.

Previous Serial Number: LBC-6

Principal Investigator: Dr. W. R. Carroll

Other Investigators: E. R. Mitchell
H. L. Wolff

Cooperating Units:

Dr. H. Eisenberg, Weizmann Institute, Rehovoth, Israel
Drs. Kohn, Rechler, Warren, Leive, LBP, NIAMD
Dr. V. Marder, CH, NIAMD
Dr. W. Jakoby, LBM, NIAMD
Dr. C. Glaudemans, LC, NIAMD
Dr. A. Dekaban, SN, NINDB
Dr. A. Schade, LM, NIAID

Man Years
Total: 3
Professional: 3
Other:

Project Description:

Objectives: The main objective of this laboratory is the understanding of the role of charged groups in determining the structure and function of proteins and other naturally occurring macromolecules. Current studies of the behavior of synthetic polyelectrolytes as simplified models are giving us some insight into the influence of charge on polymer structure as well as the possibilities of selective binding and transport of smaller ions in solution and at phase boundaries.

Methods: So far we have limited our methods to those measuring the solution properties of macromolecules. These include: ultracentrifugal analysis, light scattering, electrophoresis, diffusion, nuclear magnetic resonance, activity coefficient measurements using perm-selective membrane cells, optical rotatory dispersion, and other spectral methods.

Major Findings and Proposed Course of Studies.

Characterization of Enzymes and Bacterial Products: Sedimentation and diffusion studies for determination of heterogeneity and molecular weight, under various conditions of preparation and chemical treatment, were carried out on yeast aldehyde dehydrogenase, maleate isomerase, epoxysuccinate hydratase (Jakoby), mesotartaric acid dehydrogenase, tartronic semialdehyde reductase, glyoxylic acid reductase, oxaloglycolate reductive decarboxylase (Kohn, Rechler, Warren), milk iron binding protein (Schade), lipopolysaccharide from bacterial cell walls (Levie), S-31 immunopolysaccharide (Glaudemans), and heparitin monosulfate (Dekaban).

Identification, hydrodynamic properties, and interactions of the products of the plasmin digestion of fibrinogen: Some of the early digestion products are very similar to fibrinogen, but can be distinguished by their reactivity to thrombin and by their detailed hydrodynamic behavior, in particular, the concentration dependence of the sedimentation constant. Diffusion studies have confirmed the essential homogeneity of the isolated products and enabled the calculation of molecular weights, showing a progressive yet quite specific conversion of fibrinogen to fragments having varying biological activity and lower and lower molecular weights. (Carroll; Marder, CH)

Synthetic Polyelectrolytes: The fundamental studies of polystyrenesulfonic acid as a model polyelectrolyte are continuing, and show that large changes in dimensions take place as functions of temperature as well as ionic strength and even particular species of ion. Titration curves show that Na and K have different affinities for the polymer in solution. Such effects indicate a specificity related to size and chemistry of individual ions, and point to their particular influences on the confirmation of the polymer.

Significance to Bio-medical Research and the Program of the Institute.

Knowledge of the basic chemistry of proteins and their functions is a primary requirement for understanding physiological functions and pathological conditions of the body.

Honors and Awards: Dr. W. R. Carroll is a member of the NIH Physiological Chemistry Study Section.

Publications:

Marder, Victor J., Shulman, N. Raphael, and Carroll, William R.: The Importance of Intermediate Degradation Products of Fibrinogen in Fibrinolytic Hemmorrhage. Trans. Assoc. Am. Phys. Vol. lxxx, 156 (1967).

Serial No. NIAMD LBC-4
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The elucidation of the structure and interactions of biologically important macromolecules.

Previous Serial Number: LBC-5

Principal Investigator: Dr. H. A. Saroff

Other Investigators: Dr. R. B. Simpson
Dr. S. Dygert
Dr. E. T. Adams, Jr* (Left 9/17/66)

Cooperating Units:

Dr. R. Bloch, Weismann Inst. of Science, Rehovoth, Israel
Dr. A. Shatkay, Weizmann Inst. of Science, Rehovoth, Israel
Dr. A. Spector, LM NHI
Dr. M. Lewis, OB NINDB

Man Years
Total: 3 1/4
Professional: 3
Other: 1/4

*Present address - Illinois Inst. of Technology

Project Description:

Objectives: The main objective of this laboratory is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution and the emphasis is on the accumulation of data on the state of the molecules with respect to environment in solution. Gross features of structure as well as fine detail may be observed in the study of macromolecules in solution.

Methods: At present, we are concerned with methods of observing macromolecules in solution. In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecule, nuclear magnetic resonance (on model compounds, so far), optical rotatory dispersion measurements, and other spectral techniques. For other properties of the protein molecules, we employ the ultracentrifugal, light scattering, electrophoretic and diffusion techniques.

Major Findings and Proposed Course of Studies.

The Binding of Ions to Macromolecules: The binding of ions to proteins, synthetic polymers and model compounds is under continuing study as a probe for the determination of structure.

Studies on malonic acid and its derivatives have demonstrated that shifts in pK values in these compounds are a function of hydrogen bonds as well as electrostatic effects. Values for hydrogen bonding in water solution were determined from titration data. These values were found to be compatible with those postulated for serum albumin and ribonuclease. (Dygert, Saroff).

Data already collected in our laboratory and elsewhere on the binding of chloride ions to amines is now being evaluated to determine the nature of the perturbing effects found in the titration curves for compounds and polymers containing clusters of amino groups. (Dygert, Saroff).

A glass electrode for accurate evaluation of sodium ion activity has been constructed for study of both the properties of the glass membrane and for the binding of sodium ions to model compounds, polymers and proteins. Measurements with this electrode have reproduced results already found in this laboratory on the binding of sodium ions to malonic acid. (Dygert, Saroff).

In collaboration with the Laboratory of Metabolism of the National Heart Institute, a theoretical analysis was made of data on the distribution of fatty acids between aqueous/and non-aqueous phases, and on the binding of fatty acid anions to serum albumin. From competition studies, it was shown that the sites of strong binding for chloride are not identical with those for strong binding of fatty acid anions. (Simpson, Spector).

From spectrophotometric studies, the kinetics of exchange of copper between bovine serum albumin and ethylenediamine tetraacetic acid were determined. Since the equilibrium constant for the exchange calculated on the basis of certain assumptions varied with pH, further work on this is planned. (Simpson).

Attempts are being made to grow large crystals of bovine serum mercaptalbumin and its complex with methylmercuric ion suitable for X-ray studies to determine the three-dimensional structure of the albumin. (Simpson).

Gas chromatography of amino acids: Continued effort is being applied to the problems of analysis of amino acids by gas chromatography. Our emphasis, at present, is on a process for the formation of the derivative which can be automated. Our present sample size for starting material is about 20 nanomoles (2×10^{-8} mole) with whole sample introduced into the gas stream. (Saroff).

Significance to Bio-medical Research and the Program of the Institute:

Our emphasis is on the relationship between chemical properties and the structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the biomedical significance of our findings derive from extrapolation to the biological system.

Publications:

1. Adams, E. T., Jr.: Sedimentation equilibrium in reacting systems. V. $c_i M^2_i$ to the analysis. *Biochem.* 6: 1864-1871, June, 1967.

2. Adams, E. T., Jr., and Lewis, M. S.: Sedimentation equilibrium in reacting systems. Some applications to indefinite self-associations. Studies with β-lactoglobulin A. *Biochem.* 7: 1044-1053, Mar. 1968.

3. Bloch, Rene, Shatkay, Adam, and Saroff, H. A.: Fabrication and evaluation of membranes as specific electrodes for calcium ions. *Biophys. J.* 7: 865-877, Nov. 1967.

4. Saroff, H. A.: The binding of sodium and calcium ions to proteins. In H. Peeters (Ed.): *XVI Colloquim on Protides of the Biological Fluids.* Amsterdam, The Netherlands, Elsevier Pub. Co. 1966, pp. 45-54.

5. Simpson, R. B.: Kinetics of anion exchange between methylmercuric complexes. *J. of Chem. Phys.* 46: 4775-4783, June 1967.

Serial No. NIAMD LBC-5
1. Biophysical Chemistry
2. Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Basic mechanisms of muscular contraction.

Previous Serial Number: NIAMD LBC-2, 3

Principal Investigator: Dr. W. J. Bowen

Other Investigators: Dr. Remedios Avena
Dr. T. C. Evans, Jr. (departed 6/30/67)
Mrs. Virginia Field
Dr. Ken Hotta, Visiting Scientist

Outside Collaborator: Professor Leo Mandelkern, Florida State University

Man Years
Total: 3.5
Professional: 3.5
Other: 0.0

Project Description:

Objectives: This project is oriented toward the understanding of the events, both in energy transduction and protein transformation, of muscular contraction.

Methods: The methods used include paper and column chromatography, automated amino acid analysis, thin layer chromatography, gel filtration, paper and gel electrophoresis, UV spectrometry, ultracentrifuge analysis and isotope techniques. New techniques of isolation, detection or micro-assays were also developed.

Major Findings.

1. It has been found that ATP binds to at least two sites on both myosin B and myosin A. For myosin B, 0.4 sites of one kind and 1.2 sites of a second kind have been found per 10^5 g. These two sites have association constants (k) of 40,000 and 1500. For myosin A, these are 0.5 and 3.0 sites of the two kinds per 10^5 g with k values of 5500 and 550. Factors which convert these fractional numbers of sites per 10^5 g to integers also convert the 10^5 g unit to units of 2.5 and 2.0×10^5 g respectively, which can be considered combining (monomeric) units for myosin B and A. (Bowen, Evans)

2. Removal of C-terminal phenylalanine from actin does not affect the latter's ability to combine with myosin in so far as superprecipitation of the resulting actomyosin is concerned. It does, however, influence the rate and extent of the polymerization of actin with 0.1 M KCl - mM $MgCl_2$. (Field, Bowen)

3. Carnosine has been found to extensively enhance ATPase of myosin A at 4 mM ATP. At lower concentrations, the effect is less but definite. Most of the effect occurs during the early phases of the myosin·ATPase reaction. This is similar to the effect of carnosine on the ATP-induced contraction of glycerol-treated muscle fibers. (Avena, Bowen)

4. The effect of salts of monovalent ions on ATP-induced isotonic shortening of glycerol-treated muscle fibers seems to be one of ionic strength rather than of ionic species; but the effects of salts of divalent ions varies according to the species of cation. Mg^{++} has large accelerating effect and calcium mostly a depressing effect on shortening. Mg^{++} enhances and Ca^{++} is without effect on the initial phases of ATPase of the myofibril. (Hotta, Bowen)

5. The fast initial phase of the splitting of ATP by glycerol-treated myofibrils is greatly enhanced at about 10^{-4} M ATP. The same effect also applies to clumping (contraction) of fibrils. This coupling of fast splitting and fast contraction is striking, because at high [ATP] it does not occur. (Hotta, Bowen)

6. LiBr, which induces the shortening of glycerol-treated fibers with no other compounds present (Mandelkern, Bowen and Pebbles, Biochemistry 4, 1931, 1965) acts very much like KCl on the ATP-induced contraction, i. e., at 0.1 M it accelerates contraction. It is different from KCl in that at 10^{-5} M and 10^{-4} M it depresses both the rate of shortening and that of ATPase of homogenized glycerol-treated fibers. LiBr-induced contraction, on the other hand, is not evident below 1 Molar concentration. (Bowen, Mandelkern)

Projects proposed.

The binding of adenosine compounds is being extended to a study of the binding of pyrophosphate (PP) and inosine triphosphate. The analysis is being followed by P^{32} labeled PP. Thorough investigation of the effects of Mg^{++} and Ca^{++} is planned.

An apparent uncoupling of ATP induced shortening and myosin enzymatic splitting of ATP at high concentration will be further investigated by studies of isotonic working contractions.

Significance to Bio-medical Research and the Programs of the Institutes:

This research is oriented toward better understanding of the basic events of muscular contraction. In particular, we are interested in the transduction of ATP's energy to the contracting protein. This is coordinated with the Institute's program on metabolism and of particular concern to the cardiovascular system.

Honors and Awards:

None

Publications:

Bowen, W. J.: Methylated glycerinated muscle fibers. In Ernest, E. and Straub, F. B. (Eds.): Symposium on Muscle. Akademiai Kisdo, Budapest, 1968.

Bowen, W. J.: Effects of ouabain on ATPase and superprecipitation of myosin B. ibid.

Evans, T. C. and Bowen, W. J.: Simultaneous measurement of ATPase and superprecipitation of myosin B. Anal. Biochem. In press.

Field, V. L. S. and Bowen, W. J.: The C-terminal residue of actin and its role in reactions of actin and myosin. Arch. Biochem. Biophys. In press.

Serial No. NIAMD-LBC 6
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Clot Stabilizing Enzymes from Tissues

Previous Serial No.: NIAMD-LBC 11

Principal Investigators: K. Laki
A. Benkő, Visiting Scientist from Hungary
H. W. Thomas, Visiting Scientist from England

Cooperating Units: A. Szent-Györgyi, Institute for Muscle Research
Woods Hole, Massachusetts
J. E. Folk, NIDR

Man Years
Total: 2.5
Professional: 2.5

Project Description:

Objectives: When fibrinogen clots, two negatively charged peptides are liberated, and the thus altered fibrinogen molecules aggregate to a network structure. In the normal clotting process, a second enzyme cross-links the fibrin molecules in the clot. We have found previously that not only plasma, but also tissues, contain clot stabilizing enzymes. Since the stabilized clot is involved in wound healing, we explored whether the clot stabilizing enzyme has a role in other tissue functions.

Methods: The methods used include paper and column chromatography, automated amino acid analysis, thin layer chromatography, gel filtration, paper and gel electrophoresis, UV spectrometry, ultracentrifuge analysis and isotope techniques. New techniques of isolation, detection or micro-assays were also developed.

Major Findings: (1) Clot stabilizing enzyme in aorta: There are reports in the literature indicating that fibrin clot formation is involved in the development of atherosclerotic plaques. Since cell proliferation appears to be connected with the development of the plaques, we undertook a study to investigate whether the clot stabilizing enzyme is implicated (in analogy to its involvement in wound healing) in atherosclerosis. Rabbits were kept on high cholesterol diets and after 8 weeks, the clot stabilizing enzyme content of their aorta was compared to normal animals. A threefold increase in enzyme content was found in the aorta of the atherosclerotic animals. Also, the diseased portion of

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the aorta exhibited a threefold increase as compared to the non-diseased portion (Benkö, Laki).

(2) Clot stabilizing enzyme in muscle: We have found that skeletal muscle also contains clot stabilizing enzyme. The activity is very low; it amounts to only a few percent of the activity of liver. In connection with this finding, we observed that myosin is a substrate for clot stabilizing enzymes. The enzyme from guinea pig liver catalyzes the incorporation of amines, such as glycine ethyl ester, and putrescine into myosin. Recent experiments also indicate that this enzyme can connect myosin molecules (in a manner analogous to the cross-linking of fibrin molecules) into higher aggregates. The observation that in uterus muscle the activity of this enzyme reaches its peak just before delivery, and that the activity of this enzyme is about ten times greater in heart muscle than in skeletal muscle, indicates that this enzyme may have a role in muscular contraction. (Laki, Benkö).

(3) The structure of the clot: The alignment of the fibrin molecules in the clot is still debated. Based on the available data, a model of the clot was constructed. This model exhibits a periodicity reminding one of the pattern seen in the self-assembly of the collagen molecules. This similarity of pattern may be a partial explanation for the clot serving as a matrix for fibroblasts in wound healing. (Thomas, Laki).

Honors and Awards: None

Publications:

1. Laki, K.: The contraction of glycerol-treated fibers in Thulet's solution. Biochem. Biophys. Res. Commun. 28: pp. 103-110, June 1967.

2. Tyler, H. M. and Laki, K.: Purification and properties of a fibrin cross-linking transamidase from rabbit liver. Biochemistry 6: pp. 3259-3263, Oct. 1967.

3. Laki, K.: Review of the book: Blood Clotting Enzymology. Acad. Press, New York. Ed. Walter H. Seegers. Science 158, No. 3805, 1967.

4. Benkö, A. and Laki, K.: Studies on the clot stabilizing enzyme in aorta of rabbits under normal conditions and after cholesterol feeding. Biochem. Biophys. Res. Commun. 31: No. 2, pp. 231-240, 1968.

5. Chandrasekhar, N. and Laki, K.: Evolution of the fibrinogen-thrombin interaction. In Laki, K. (Ed.): Fibrinogen. New York, London, Marcel Dekker, 1968, pp. 117-130.

Serial No. NIAMD-LBC 7
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Actin, the primary energy transducer of muscle.

Previous Serial No.: None

Principal Investigators: K. Laki and R. E. Alving

Cooperating Units: None

Man Years
Total: 1-1/4
Professional: 1-1/4

Project Description:

Objectives: Muscle is an energy transducer in which chemical energy is converted to work. So far, no satisfactory explanation exists on how the energy liberated from ATP is coupled to mechanical ev ents. It is apparent that in the primary transducer the decomposition of ATP must be coupled to mechanical events. In other words, we need an ATPase which dephosphorylates ATP only when strictly coupled to mechanical events. F-actin is such an ATPase: It splits ATP molecules when proper mechanical deformation is applied. The purpose of this project is to explore the details of the mode of action of this "mechano-chemical" enzyme.

Methods: Methods applied in this project involved the use of sonicator, tritium and C^{14}-labelled materials, scintillation counters, paper and column chromatography, and others.

Major Findings: F-actin, which normally contains firmly bound ADP, is known to behave as an ATPase under the influence of ultrasonic vibration. The specificity, the high binding energy, and the profound effect of adenosine nucleotides on actin, strongly suggest a covalent rather than ionic type of bond between these molecules. Our investigations of the nature of this bond indicate participation of at least one of the sulfhydryl groups of actin in this bond. N-ethyl maleimide (NEM), which under normal conditions reacts with only two sulfhydryls in actin, will react with at least one additional SH group when the nucleotide is released by ultrasonic vibration. In addition, we find that ATP, but not ADP, is a powerful inhibitor of NEM incorporation during sonication. Actin continues to react slowly with NEM after sonication, but addition of ATP will stop this reaction. Only the NEM which is incorporated into actin during sonication has an inhibiting effect on its ATPase activity. This occurs when about

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two moles have reacted. Experiments are in progress to determine whether the binding of ATP is inhibited by the incorporation of NEM during sonication and preliminary results indicate that this is the case. In contrast to the effect of NEM, similar experiments with DFP showed no inhibitory effects.

These experiments suggest that in the "active center" of actin, a cysteine residue reacts with ATP in such a manner that phosphate is released and the rest of the ATP molecules "acylate" the enzyme. In the final step, mechanical deformation promotes the acyl-intermediate (adenosine-diphosphoryl-actin) to release the nucleotide as ADP.

Proposed course of project: Experiments are in progress to examine 0^{18} incorporation into ADP accumulating when actin is under sonication. Also, attempts will be made to see if other compounds such as nitrophenylacetate would also act as a substrate.

Honors and Awards: None.

Publications: None.

Serial No. NIAMD-LBC 8
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biochemical studies on unusual amino acids and metabolites in living systems.

Previous Serial No.: NIAMD-LBC 8

Principal Investigator: Filadelfo Irreverre

Technican Assistance: Mr. Edwin Wilson

Cooperating Units: Dr. L. Laster, MDB, NIAMD
Dr. S. H. Mudd, LGCB, NIMH
Dr. A. K. Percy, DIR, NINDB
Dr. H. Shichi, O, NINDB
Dr. M. Lewis, O, NINDB

Man Years
Total: 2
Professional: 2

Project Description:

Objectives: Using tools developed in this laboratory, such as high resolution 2-dimensional paper chromatography coupled with ion-exchange chromatography and high voltage paper electrophoresis, several new amino acids and interesting metabolites have been discovered, isolated, and characterized (covered in previous reports). In addition, many unknown compounds have been observed in physiological fluids and tissue extracts from various sources including man. Studies have been directed towards the isolation and characterization of some of these metabolites and tracing of their biochemical pathways in the living organism.

Methods: Modern techniques in biochemistry have been employed. These are: paper and column chromatography, automated amino acid analysis, thin layer chromatography, gel separation and filtration, paper and gel electrophoresis, UV, infrared and mass spectrometry, NMR, polarimetry, ultracentrifuge, isotope techniques and enzymatic analysis. When necessary, development of new techniques of isolation, detection or microassay has been undertaken.

Major Findings: The isolation and identification of S-sulfo-L-cysteine from the urine of a mentally retarded child with severe brain damage and dislocated lenses resulted in the discovery of a new disease -

sulfite oxidase deficiency (covered in last year's report). Continued studies were made on this interesting abnormality in man.

Since a patient with sulfite oxidase deficiency has a lack of inorganic sulfate, and since the latter is the ultimate precursor of the organic sulfate esters in such materials as myelin, cartilage and acid mucopolysaccharides, it is a possibility that this deficiency would be reflected in the tissues which in turn might contribute to development of the pathological changes in the patient.

To evaluate this hypothesis, studies were undertaken towards 3 parameters - sulfatides or sphingoglycolipids, tyrosine-0-sulfate and indoxyl-3-sulfate. Measurements of these compounds in tissues and physiological fluids might yield an explanation to the biochemical abnormalities found in the patient's urine.

1. Sulfatides or sphingoglycolipids: Extraction, characterization and quantitative determination of sulfatides from the patient's brain and kidney were carried out. The results showed values of sulfatides comparable to those of normals suggesting that the patient did have enough inorganic sulfate available for formation of some myelin. However, it should be noted that his brain weighed only 470 g., whereas the normal weight for a child this age is approximately 1000 g., and that histologic studies (to be reported elsewhere by Dr. W. I. Rosenblum, NINDB) revealed a diffuse deficiency of myelin. Thus, the total brain content of sulfatide was below that expected. The question of whether this reduction was due to deficiency of inorganic sulfate or to some other process responsible for the general brain damage is not answered by the present study.

2. Tyrosine-0-sulfate: This compound was isolated, characterized and determined quantitatively in the patient's urine. The values found are comparable to those of control children similarly treated.

3. Indoxyl-3-sulfate: This compound was also isolated, characterized and quantitatively determined in the patient's urine. The values obtained when subjected to statistical analysis showed significant deviation from normal values. They were lower.

Although 2 out of the 3 parameters studied, namely, the sulfatides in brain and kidney and tyrosine-0-sulfate in urine, gave normal values, these do not prove their biosynthesis was proceeding at a normal rate. Even a reduced rate of synthesis could lead to normal values if the rate of degradation were less than normal. Sulfatase A, the enzyme which may be responsible for normal sulfate ester degradation, is strongly inhibited by sulfite. The possibility of sulfite inhibition of the degradation of sulfate esters should be considered in future investigations of sulfite oxidase deficiency. (A. K. Percy, S. H. Mudd, F. Irreverre, and L. Laster).

An interesting acidic peptide containing a few amino acids has been isolated from the urine of the same patient with sulfite oxidase deficiency. The amino acid sequence is being determined. (F. Irreverre).

Rhodopsin, a glycoprotein from bovine source, has been prepared, purified and its amino acid content determined. It was shown for the first time that glucosamine is present. (H. Shichi, M. Lewis and F. Irreverre).

Significance to Bio-medical Research and the Program of the Institute: Studies of metabolites and macromolecules and tracing their roles in the complex living system, specially in man, should give us added knowledge for a better understanding of health and disease.

Proposed course of project: Biochemical studies on unusual amino acids and metabolites in living systems will continue. Special emphasis will be directed towards the isolation and identification of some of the unknown compounds in physiological fluids from mentally retarded children which were uncovered in previous studies.

Honors and Awards: None

Publications:

1. Mudd, S. H., Irreverre, F. and Laster, L.: Sulfite oxidase deficiency in man: Demonstration of the enzymatic defect. Science 156: 1599-1602, June 1967.
2. Irreverre, F., Mudd, S. H., Heizer, W. D. and Laster, L.: Sulfite oxidase deficiency: Studies of a patient with mental retardation, dislocated ocular lenses, and abnormal urinary excretion of S-sulfo-L-cysteine, sulfite, and thiosulfate. Biochem. Med. 1: 187-217, Nov. 1967.

Annual Report of the Laboratory of Molecular Biology

National Institute of Arthritis and Metabolic Diseases

During the last year the Section of Molecular Structure, under the direction of Dr. David Davies, has continued its studies of the structure of gamma chymotrypsin. A 5.5 Å electron density map has been obtained and for purposes of comparison, a model of alpha chymotrypsin has been constructed. Several significant differences between alpha and gamma chymotrypsin have been found having to do with the position of tyrosine 146, located near the active center of the molecule. This residue appears to be more buried in the alpha than the gamma form of the enzyme. In addition, in comparing different structures between tosyl and native molecules in the alpha and gamma forms of chymotrypsin, a hole was observed in the gamma molecule which does not occur in the alpha. This also seems to be due to the difference in the position of tyrosine 146. (D. R. Davies, G. Cohen, B. Matthews, E. Silverton, and H. Braxton)

Work in the Section on Physical Chemistry has continued along several lines. In a continuing study on the interaction of DNA with basic polypeptides in histones, it has been discovered that the interaction of DNA with polylysine results in the formation of micelles of uniform size detected by light scattering and sedimentation velocity experiments. The preference of polylysine for A-T rich DNA is reversed by tetramethylammonium ion so that G-C rich DNA is selected instead. These studies are being extended to nucleohistones derived from biological sources and are intended to aid in the ultimate understanding of the regulation of gene activity. (G. Felsenfeld, J. T. Shapiro, F. Bernardi, and B. J. Sebring)

Other studies have been directed toward the understanding of the structure of polymers of uridylic acid and adenylic acid. The two single stranded molecules have different secondary structure, the former not being stacked where the latter probably is. Theoretical calculations, based on restriction of bond angles and freedom of rotation, have been attempted in order to compare actual polymer conformation with theoretical possibilities. Since single stranded polynucleotides function as messenger RNA, these studies provide an important physical understanding of their structure and are the first of their kind to use classical polymer methods. (G. Felsenfeld, and L. D. Inners)

Further studies have been carried out using microcalorimetry to evaluate the energetic contributions of various chemical components of nucleic acids to the thermodynamic stability of nucleic acid helical complexes. (P. D. Ross, and R. L. Scruggs)

Studies on the DNA lygase reaction have continued and the presence of an adenylated enzyme intermediate in the reaction have been found. The mechanism of the reaction, which involves DPN, as well as an enzyme, and nicked DNA, has been worked out. The enzyme provides one of the best models for the understanding of DNA repair, although its exact physiological role is not yet known. (M. Gellert, S. B. Zimmerman, J. W. Little, and C. K. Oshinsky)

In the Section on Microbial Genetics, the sex factor, F, and bacteriophage P1 have been found to exhibit the parallel behavior in causing the induction of lysogenic bacteria. Conversely, lambdoid phages tested did not behave like F either in causing induction of lysogenic bacteria or in cosegregating with the bacterial chromosome. In addition, a variety of alternative modes of prophage integration and excision have been analyzed with bacterial and phage mutant defective in various recombination functions and in attachment regions. Conditions have been found in which each function plays an important role in either excision or lysogenization. (M. Yarmolinsky, M. Gottesman, and J. L. Rosner)

Genetic studies have been done on fused operons and a transport deficient Salmonella mutant. A detailed study of deletion mutants, with fused histidine and rough b operons, in Salmonella typhimurium is being made. These operons are transcribed in opposite directions so that there is a symmetrical competitive interference between the two operons which is a function of their relative rates of transcription. The biochemistry and genetics of carbohydrate transport deficient mutants are also being studied. Two separate classes of mutants which lack different components of the phosphotransferase system have been found. (M. Levinthal)

In studies on biochemical control mechanisms in histidine biosynthesis, a further analysis of other genes in histidine operons has shown that the original translation model of polarity requires modification. Apparently at least two parameters are involved in determining the extent of polarity exerted by a nonsense mutant. The possible involvement of mRNA synthesis in polarity is being investigated, but any possible relationship between polarity and relaxation has been disproven. A genetic locus co-transducible with the histidine operon has finally been isolated and its exact map position is being determined. In addition, the effect of regulated gene dosage on control of histidine operon is being investigated. (R. G. Martin, M. Bagdasarian, D. Gutnick, H. J. Whitfield, Jr., and B. Ames)

In the Section on Chemical Genetics, two problems have been pursued. One is a continuation of the study of the control of hemoglobin synthesis. The difference between delta chain and beta chain synthesis has been shown to be probably due to a difference in the concentration of messenger RNA for the two proteins as well as a possible difference in the rate at which the messengers are translated. Additional studies on gamma globulin of mouse myeloma have been initiated and an in vitro system where labeling of the light chain occurs has been developed. (H. A. Itano, W. M. Fogarty, Jr., H. H. Kazazian, Jr., T. Seki, E. R. Tudor, and E. N. Verrusio)

In the Section on Organic Chemistry, 5' GMP and 5' DGMP have been found to interact stoichiometrically with poly C and polybrom C to form two stranded helical structures. The ribocomplexes are more stable than the ribodeoxy complexes and the brom C complexes are more stable than the C complexes. The complex formed between poly I and polybrom C has been studied by means of infrared and ultraviolet spectroscopy, optical rotatory dispersion and circular dichroism. In addition, studies showing that the non-enzymatic synthesis of polynucleotides can to some extent be directed by other polynucleotides, make it appear as though it might be important in understanding the origin of living systems. (H. T. Miles, F. B. Howard, K. Ikeda, and J. Frazier)

In the Section on Metabolic Enzymes, three projects have been studied. First, continuation on the mechanism of enzyme induction in mammalian cells in tissue culture by steroid hormones. A transcription-translation control model has been proposed with the steroid hormone inducer involved in controlling both processes. The chemical structure of the inducible tyrosine aminotransferase has been studied and a direct search has been made for the RNA fraction whose synthesis is stimulated by the hormone. Studies on synchronized cell populations have revealed that certain phases of the cell cycle behave differently from other phases with respect to enzyme inducibility and preliminary chemical investigations have been begun on hormone binding proteins isolated from tissue culture cells. (G. Tomkins, E. B. Thompson, T. Gelehrter, F. Auricchio, F. Valeriote, R. Gardner, H. Samuels, D. W. Martin, Jr., and D. K. Granner)

The second project has been the study of the mechanism of cysteine biosynthesis and its control in bacteria. Two enzymes, serine transacetylase and o-acetyl serine sulphydrylase, have been isolated in a nearly homogeneous state. Second enzyme appears to be composed of two identical subunits each weighing about 32,000. The first enzyme is probably a tetramer of identical subunits which is associated with a small amount of the dimeric o-acetyl serine sulphydrylase. The substrate of one of the reactions, o-acetyl serine, causes a disaggregation of the complex enzyme molecule. Current investigations involve the genetic control of cysteine biosynthesis and the possible role of changes in the protein conformation in the regulation of enzyme biosynthesis. (N. Kredich, M. Becker, and G. Tomkins)

A third project has been the study of a mechanism of protein synthesis in mammalian tissues. Preliminary studies on a chain initiation in mammalian cells have suggested that the mechanism may be different from that found in bacteria, that is it may not involve formylmethionine. Other studies have confirmed the bacterial requirements for the different functions of various factors in protein synthesis, but the detailed mechanism in mammalian cells, which may be different, is under active investigation. (E. Maxwell, S. Raeburn, R. Goor, and J. Schneider)

Serial No. NIAMD-LMB-1
1. Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzyme induction in tissue culture cells

Previous Serial Number: NIAMD-LMB-3

Principal Investigator: Gordon Tomkins

Other Investigators: E. B. Thompson, Thomas Gelehrter, Ferdinando Auricchio, Guest Worker, F. Valeriote, Guest Worker, Robert Gardner and Herbert Samuels

Cooperating Units: None

Man Years

Total:	7-1/2
Professional:	7-1/2
Other:	0

Project Description:

Objectives: The control of gene activity in mammalian cells is at the center of cell differentiation. Over the past few years, we have developed a system in which a steroid hormone activates the synthesis of a specific gene product in tissue culture cells, and we have been investigating the mechanism of this interaction, which should give insight into both the regulation of gene expression in mammalian cells as well as the mechanisms of hormone action.

Methods employed: The methods employed are those of enzymology, protein chemistry and tissue culture.

Major findings: 1. Structure of tyrosine aminotransferase. The inducible enzyme is tyrosine aminotransferase, which has been highly purified from rat liver by our group, and its sub-structure has been investigated. It appears to be a pyridoxal protein composed of 4 identical subunits, each weighing about 30,000. The protein can be obtained presently in high yield and at high purity by relatively simple purification scheme. Studies of the primary sequence of the protein have just begun, but peptide mapping indicates that the protein is composed of similar or identical subunits.

2. Regulation of tyrosine transaminase synthesis by serine. It has been discovered that the serine level in the growth medium of tissue culture cells can control their concentration of tyrosine aminotransferase. Serine, in high concentration, appears to repress the synthesis of the enzyme, while at low concentration the synthesis is increased. The mechanism of the serine effect is currently under investigation and its relation to steroid hormone induction is being actively pursued.

3. Steroid receptor proteins. A soluble, relative low molecular weight protein component has been identified and partially purified from extracts of tissue culture cells which formed strong, noncovalent bonds with inducing steroids. The relationship of this to the process of enzyme induction is currently under investigation.

4. Superinduction. Previously we had found that high concentrations of Actinomycin D given to the tissue culture cells cause an increase in the level of tyrosine aminotransferase under certain circumstances. We have traced this increase to an increase in the rate of enzyme synthesis and as a result of this, as well as several other experiments, have proposed a mechanism whereby the hormone regulates enzyme formation by controlling both gene transcription and messenger RNA translation.

5. Messenger RNA. Previous evidence had indicated that messenger RNA accumulates on enzyme induction and the chemical search for this RNA fraction has been made. Very small, but probably significant increases in the synthesis of the specific RNA's have been detected on sucrose gradients and by acrylamide electrophoresis of various RNA fractions.

Significance to the program of the Institute: The role of hormones in controlling biological processes as well as the control of gene activity are both obviously very closely related to the objectives of NIAMD research, namely an understanding of the chemical basis of inherited metabolic diseases.

Proposed course of project: We plan to continue the study and the structure of the enzyme, the steroid binding protein and try to determine the precise mechanism by which the hormone exerts its action on the synthesis of the protein. In addition, we are beginning to attempt genetic studies in mammalian cells designed to provide mutants of the enzyme as well as possibly regulatory mutants.

Publications:

Hayashi, S., Granner, D. K., and Tomkins, G.M.: Tyrosine aminotransferase: Purification and characterization. J. Biol. Chem. 242: 3998-4006, Sept. 1967.

Peterkofsky, B. and Tomkins, G.M.: The effect of inhibitors of nucleic acid synthesis on steroid-mediated induction of tyrosine amino-transferase in hepatoma cell cultures. J. Mol. Biol. 30, 49-61, Dec. 1967.

Loeb, J. N., Howell, R. R. and Tomkins, G. M.: Free and membrane-bound ribosomes in rat liver. J. Biol. Chem. 242, 2069-2074, Sept. 1967.

Gelehrter, T. D. and Tomkins, G. M.: The role of RNA in the hormonal induction of tyrosine aminotransferase in mammalian cells in tissue culture. J. Mol. Biol. 29, 59-76, Oct. 1967.

Eisenberg, H. and Tomkins, G. M.: The molecular weight of the subunits, oligomeric and associated forms of bovine liver glutamate dehydrogenase. J. Mol. Biol. 31, 37-49, Feb. 1968.

Peterkofsky, B. and Tomkins, G. M.: Evidence for the steroid-induced accumulation of tyrosine-aminotransferase messenger-RNA in the absence of protein synthesis. PNAS, in press.

Serial No. NIAMD-LMB- 2
1. Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Amino acid incorporation into protein *in vitro*: Mechanisms and possible metabolic controls.

Previous Serial Number: NIAMD-LMB-5

Principal Investigator: Elizabeth S. Maxwell

Other Investigators: Samuel Raeburn, Ronald Goor, and Jerry Schneider

Cooperating Units: None

Man Years

Total:	4
Professional:	4
Other:	0

Project Description:

Objectives: We are investigating protein synthesis in cell-free mammalian systems. An understanding of polypeptide chain initiation, elongation and completion at the enzymatic level is fundamentally important and interesting of itself. An added incentive to our studies lies in the possibility that fresh approaches to the problems of growth and differentiation may come to light through such understanding.

Methods employed: All of the methods used are either standard procedures or minor modifications thereof.

Major findings:

1. Initiation of polypeptide chains: Recently a great deal of information has been discovered in other laboratories concerning the mechanism by which the initial peptide bond is formed. In *E. coli* each polypeptide chain begins with N-formylmethionine. Formation of the first peptide bond requires several soluble protein factors, GTP and N-formylmethionyl T-RNA in addition to the ribosome-messenger complex. We have looked for evidence of a similar mechanism in several mammalian systems including reticulocytes, rat liver and, with Dr. Brad Thompson, a line of hepatoma cells in tissue culture. We have used inhibitors of dihydrofolic reductase which, in similar experiments in *E. coli*, inhibit protein synthesis by preventing the formation of N-formyl-

methioninyl T-RNA. All our data indicate that initiation in mammalian systems probably proceeds by a different mechanism.

2. Elongation of polypeptide chains: Once initiated, the growth of polypeptide chains on mammalian polyribosome-messenger RNA complexes requires, in addition to GTP and amino-acyl T-RNA, two soluble protein factors designated transferases I and II. The process is believed to proceed in at least three steps: 1) binding of aminoacyl T-RNA, 2) peptide bond formation, 3) either movement of messenger or translocation of peptidyl T-RNA back to a donner or reactive site. Until recently the role of the soluble factors in the overall process was unknown. We developed an assay using H^3 puromycin which we believe measures peptide bond synthesis per se and subsequently detects translocation. Peptide bond formation occurs without the addition of GTP and is catalyzed by well-washed polysomes. Translocation of peptidyl T-RNA requires GTP and is catalyzed by transferase II. The data are consistent with recent results in bacterial systems which indicate that peptide synthetase is an integral part of ribosomes and that translocation requires at least one soluble enzyme and GTP.

Significance to NIAMD research: The enzymatic mechanisms in protein synthesis in mammals and the metabolic control of these processes is of major interest to biology and biochemistry. Our emphasis so far is on enzymatic mechanisms. Understanding of control will follow.

Proposed course of project: We propose to continue investigation of the enzymatic steps involved in peptide chain initiation and extension in mammalian systems. The possibility of metabolic control at each step investigated will be considered.

Serial No. NIAMD-LMB-3
1. Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Synthesis of Tyrosine Aminotransferase in Tissue Culture

Principal Investigator: Daryl K. Granner

Other Investigators: E. Brad Thompson
Gordon M. Tomkins

Cooperating Units: none

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives: We succeeded in preparing a highly specific antiserum against purified tyrosine aminotransferase (TAT) for the purpose of studying factors controlling the synthesis of this enzyme in tissue culture. It was our specific objective to define the nature of the induction of TAT by dexamethasone phosphate, a synthetic glucocorticoid hormone.

Methods employed: Modifications of standard immunochemical methods were employed.

Major findings: We have shown that TAT induction is due to an increased rate of synthesis of the protein from free amino acids. As part of these studies, we have shown that both basal and induced TAT turnover ie are constantly being both synthesized and degraded. Although the HTC cells are capable of synthesizing TAT at induced levels for 40-48 hours, constant presence of steroid is required. Since the same holds when ribonucleic acid synthesis is inhibited, we have suggested a steroid control on translation of the genetic message.

Significance to NIAMD research: Specific features of the control of protein synthesis in mammalian cells are not known. Furthermore, the manner in which steroid hormones function at the cellular level is not known. We feel the study of TAT synthesis in this hepatoma tissue culture system affords a good model for such investigations.

Proposed course of project: The rates of synthesis and degradation of TAT are going to be studied in greater detail as are the effects of various hormones and metabolic inhibitors in these functions. The possible translational control of protein synthesis by dexamethasone will be investigated.

Publications:

Tomkins, G. M., Gelehrter, T. D., Granner, D. K., Peterkofsky, B., and Thompson, E. B.: Regulation of gene expression in mammalian cells. In Hurlbert, R. B. (Ed.): Exploitable Molecular Mechanism and Neoplasia. In press.

Granner, D. K., and Tomkins, G. M.: Tyrosine aminotransferase. In Colowick, S. P., and Kaplan, N. O. (Eds.): Methods in Enzymology. New York, N. Y., Academic Press. In press.

Granner, D. K., Hayashi, S., Thompson, E. B., and Tomkins, G. M.: Stimulation of tyrosine aminotransferase synthesis by dexamethasone phosphate in tissue culture. J. Mol. Biol. In press.

Serial No. NIAMD-LMB- 4
1. Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Hepatoma Tissue Culture Cell Cycle Analysis.

Previous Serial Number: None

Principal Investigator: Dr. D. W. Martin, Jr.

Man Years

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: To resolve the mammalian tissue culture cell generation cycle and the dependence of the TAT gene expression upon the specific phases of the cycle.

Methods employed: Mammalian tissue culture, synchronization of mammalian cells, radioactive isotopic labelling. Immunologic assays, polyacrylamide gel electrophoresis, pulverization of acrylamide gels, computer processing of data.

Major findings: The generation cycle of the hepatoma tissue culture (HTC) cells can be divided into four phases. The morphologically distinct mitosis might be considered the starting place, being of some sixty to ninety minutes in duration. Mitotic division is followed by a gap (G1) of 10 hrs prior to the beginning of DNA synthesis (S phase). The latter persists nine hours and is succeeded by a second gap (G2), a 4-5 hr period, separating it from the recycle mitosis. Using random populations of HTC cells it has been previously shown that exposure to corticosteroids effects a ten fold increase in the enzyme tyrosine aminotransferase (TAT) specific activity. The resolution of the cell cycle allowed us to examine the expression of the TAT gene in the described phases both basally and in response to the steroid inducer.

Although TAT protein can be translated in mitosis, an increased rate of synthesis cannot be induced by the steroid. Several hours after entering into G1 the cell becomes capable of synthesizing TAT at an increased rate. Such capability of induction persists through S phase but is not present during the G2 phase. Thus there is a period extending from the end of DNA synthesis through the completion of mitosis and several hours into G1 during which the TAT gene cannot, in response to inducer, be expressed at an increased rate.

Significance to Bio-medical Research and the Program of the Institute: Control over expression of both regulatory and structural genes can be effected through the specific phases in the generation cycle of the mammalian cell. This control provides a powerful mechanism by which cellular processes such as differentiation, malignancy, and aging might occur.

Proposed course: To further investigate the mechanisms of phase control and its applicability to both physiologic and pathologic processes.

Serial No. NIAMD-LMB-5
1. Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Cysteine biosynthesis in Salmonella typhimurium

Previous Serial Number: NIAMD-LMB-6

Principal Investigators: Nicholas Kredich, Michael Becker and
Gordon Tomkins

Other Investigators: None

Cooperating Units: None

Man Years

Total:	2-1/4
Professional:	2-1/4
Other:	0

Project Description:

Objectives: To understand the biosynthesis of cysteine and its regulation in Salmonella typhimurium. The genes of this pathway are in separate clusters on the Salmonella chromosome and therefore the regulation is rather complex, one group of genes influencing the activity of another. In addition, the system may display both negative and positive regulation, which makes it an excellent one in which to study regulatory genetics.

Methods employed: Methods employed have been standard enzymological and microbial genetic techniques.

Major findings: Cysteine biosynthesis proceeds by a two-step reaction: 1) Serine + Acetyl Co A $\longrightarrow$ O-acetyl serine;2)O-acetyl serine + SH- $\longrightarrow$ cysteine. The second enzyme (II) has been purified to near homogeneity and found to be a dimer composed of identical subunits, each weighing about 32,000. The enzyme is a pyridoxal protein. The first enzyme (I) has also been highly purified. It forms a complex with a small amount of enzyme (II) which is capable of catalyzing the formation of cysteine from acetyl CoA, serine, and sulphide. This complex is called cysteine synthetase (CS). Cysteine synthetase can be dissociated into its component parts, enzyme (I) plus enzyme (II), in the presence of O-acetyl serine. Recent genetic evidence obtained by Pasternak in England has shown that the cysE gene which codes for enzyme (I) of the cysteine synthetase complex is required for the synthesis of the enzymes leading from sulfate to sulfide, thus acting as a positive regulator. We are presently investigating whether the protein dissociation reaction is related to the regulation.

Significance to the program of the Institute: The control of gene activity is a fundamental biological mechanism which breaks down in many disease states. The type of regulation which occurs in the cysteine pathway is sufficiently different from other systems which have been worked out that we feel there may be a new principle of gene regulation.

Proposed course of project: We plan to continue investigating both the genetics and structure of the enzymes of cysteine biosynthesis.

Publications:

Kredich, Nicholas M. and Tomkins, Gordon M.: The Biosynthesis of l-Cysteine in *Escherichia coli* and *Salmonella typhimurium* by a multi-functional Enzyme Complex. In Vogel, H. J., Lampen, J. O. and Bryson, V. (Eds.): *Organizational Biosynthesis*. Academic Press Inc., New York, 1967, pp. 189-198.

Serial No. NIAMD-LMB-6
1. Molecular Biology
2. Chemical Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemistry and genetics of hemoglobin and other proteins.

Previous Serial Number: NIAMD-LMB-7

Principal Investigator: Harvey A. Itano

Other Investigators: Dr. William M. Fogarty, Jr., Dr. Haig H. Kazazian, Jr., Dr. Tokuichiro Seki, Mrs. Elizabeth R. Tudor, and Mrs. Elaine N. Verrusio.

Cooperating Units: Dr. Ettore Appella (NCI-3229).

Man Years	
Total:	7
Professional:	5
Other:	2

Project Description:

Objectives: To study the genetic control of the structure and rate of synthesis of proteins and to study the chemistry and function of proteins.

Methods employed: Characterization of proteins, peptides, and amino acids by chromatography, electrophoresis, ultracentrifugation, and spectrophotometry. Chemical modification of amino acids and proteins. Synthesis, isolation, and characterization of derivatives of amino acids by the methods of organic chemistry. Study of protein biosynthesis with radioactive amino acids, gradient centrifugation, and the methods of peptide analysis.

Major findings: A normal human erythrocyte contains 40 times as much β chain of hemoglobin A ($\alpha_2\beta_2$) as δ chain of hemoglobin A_2 ($\alpha_2\delta_2$). The basis of this difference was investigated with the use of reticulocytes from individuals with sickle cell anemia, whose erythrocytes contain 30 times as much β^S chain of hemoglobin S ($\alpha_2\beta_2^S$) as δ chain. Ribosomes and supernatant hemoglobins were isolated from reticulocytes that had been incubated with ^{14}C-labeled amino acids. Peptides obtained from tryptic digests of incomplete, or nascent, polypeptide chains attached to ribosomes and globin were purified and assayed for radioactivity. The nascent chains of the ribosomal preparations contain 0.01-0.02 as much δT1 and δT3 as β^ST1 and β^ST3. Thus, the number of active δ-chain-associated ribosomes is assumed to be 0.01-0.02 that of β^S-chain-associated ribosomes. The labeling of tryptic peptides from supernatant hemoglobins A_2 and S indicated that the rate of δ chain synthesis is 0.005 that of the β^S chain. Upon solving the appropriate equation, we found that the translation rate of β^S mRNA may be 2 to 4 times faster than that of δ mRNA. However, these data suggest that a lower concentration of δ chain mRNA is the major cause of the slower rate of δ chain synthesis. In vitro incubation experiments suggest that δ mRNA is not more unstable than β^S mRNA. Thus, it is likely that the overall synthesis of δ mRNA is less than that of β^S mRNA. As presently understood, an operon of δ and β chain genes would require that the synthesis of δ mRNA be no less than that of β^S mRNA. These data provide evidence against the existence of such an operon.

Methods were developed for the isolation and purification of an immunoglobulin light chain product of a mouse myeloma. An in vitro system for incorporation of radiolabeled amino acids into the protein was developed and studies on methods to recover nascent peptides from the cells after short term incubations are in progress. These timed studies will be used to study the synthesis rate of the protein and are part of a series of studies on the relationship of sequence to synthesis rate in proteins.

The structural analysis of γA mouse myeloma protein (47A) was investigated. This protein can be isolated from urine of Balb/c mice by chromatography on DEAE-cellulose. The purified product consisted of two molecular species, a monomer with a sedimentation constant in the ultracentrifuge of 3.9 S having one heavy and one light chain and a dimer with S value of 5.7. The heavy and light chains of the monomer can be separated by a column of Sephadex G-100 in N acetic acid containing 8 M urea after partial reduction and carboxymethylation. Glutamine was found as carboxyl terminal amino acid of the monomer heavy chain. The γA heavy chains isolated from sera of different mouse tumors have tyrosine at the carboxyl terminus. In these heavy chains, the second amino acid residue after tyrosine has been reported to be cysteine. So far, in monomer heavy chain, no evidence was obtained as to the existence of cysteine at the second residue from carboxyl-terminal end. A plausible hypothesis at the present time is the existence of a deletion at the carboxyl terminal of monomer heavy chain. The extent of this deletion is not yet known.

In order to prevent tryptic digestion of lysyl and aminoethylcysteinyl bonds and at the same time preserve the basicity of these residues, complete guanidination of aminoethylated protein was tried. A new amino acid, 4-thiahomoarginine, which is formed by guanidination of aminoethylcysteine residue, was synthesized.

The nature of the reaction of 1,2-diketones with mono- and 1,1-disubstituted guanidines was investigated. A number of condensation products were synthesized and were shown to be new compounds. The reaction of phenanthrenequinone with monosubstituted guanidines was found to produce an aldehyde in addition to the fluorescent product previously reported.

Proposed course of project: Studies on chemical modification of amino acids and proteins, on genetic control of structure and rates of synthesis of proteins, and on the chemistry and genetics of hemoglobin will be continued. The relationship between structure and rate in the synthesis of proteins will be studied with the use of mouse myeloma globulins as well as with hemoglobin.

Significance to the program of the Institute: The objectives of the program are to inquire into the genetic control of protein synthesis and to study the relation of the structure of a protein molecule in its function. Such studies are likely to furnish information applicable to understanding the effect of inherited defects in protein structure on metabolic disease.

Publications:

Itano, H. A.: The structure-rate hypothesis and the toll bridge analogy. In Rich, A., and Davidson, N. (Eds.): Structural Chemistry and Molecular Biology. San Francisco and London, W. H. Freeman and Company, 1968, pp. 275-280.

Kazazian, H. H., Jr., and Itano, H. A.: Studies on the quantitative control of polypeptide synthesis in human reticulocytes. J. Biol. Chem. 243: 2048-2055, April 1968.

Serial No. NIAMD-LMB-7
1. Molecular Biology
2. Organic Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemical and Structural Investigations of Nucleic Acids and Related Substances.

Previous Serial Number: NIAMD-LMB-8

Principal Investigator: H. Todd Miles

Other Investigators: Dr. F. B. Howard, Dr. K. Ikeda, and Mr. J. Frazier.

Cooperating Units: Dr. E. D. Becker and Dr. R. R. Shoup (NIAMD-LPB-13);
Drs. L. Orgel, J. Sulston, and R. Lohrman, Salk Institute,
San Diego.

Man Years

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives: Basic chemical, biochemical, and structural studies of nucleic acids and related substances.

Methods: Infrared spectroscopy, nuclear magnetic resonance, spectroscopy, chemical synthesis and structure elucidation, optical rotation, ultraviolet spectroscopy, use of enzymes for synthesis and structure study.

Major findings: We have found conditions for complete stoichiometric interaction of ribo and deoxyribo guanylic acids with poly C and poly bromo C to form two-stranded helices (previously we have reported complete interaction to form three-stranded C_2G helices and incomplete formation of two-stranded helices). Infrared spectroscopy has been invaluable in these studies in demonstrating both the specificity of interaction and the absence of any of several other possible hetero and homo complexes. Whereas the adenosine and deoxyadenosine complexes with poly U had shown no difference in Tm, the complexes formed by 5'-GMP and 5'-dGMP with poly C and with poly BC did show consistent differences in stability, the rGMP complexes being more stable. Preliminary studies of concentration dependence and of salt dependence of these four GC complexes have been carried out.

These new complexes provide good infrared catalog spectra for analysis of base pairing in nucleic acids.

Oxygen-18 substitution of GMP in the complexes has permitted unambiguous assignment of G carboxyl vibrations in IR spectra of the two-stranded GC helices and has provided evidence of vibrational coupling between G and C in the helices.

5'-GMP and 3'-GMP are known to form ordered, helical structures of high viscosity in acid solution (pH 5) but were reported not to form such structures in neutral solution (Gellert, Lipsett, and Davies, *Proc. Nat. Acad. Sci.* (1962)). We have found that 5'-GMP does form an ordered structure at pH 7.5 but that, unlike those previously reported, it does not form a viscous gel. The IR spectra and optical rotation are consistent with formation of a structure similar to that formed by 3'-GMP in acid solution (pH 5). The additional electrostatic repulsion of a second negative charge on each nucleotide phosphate is presumably responsible for the failure to form three dimensional aggregates leading to gel formation. NMR observations suggest that aggregates formed by 5'-GMP at neutral pH are not of high molecular weight.

5-Bromocytidine-5'-pyrophosphate has been synthesized by direct bromination of the pyrophosphate in formamide (method of Lipkin and Markham). In addition to the desired product a saturated non UV-absorbing impurity was demonstrated by C^{14} labelling but was shown not to be a substrate for polynucleotide phosphorylase.

Poly $\overline{BC}$ was prepared enzymatically and the properties of its interaction product with poly I investigated by IR, UV, ORD and CD. Introduction of the 5-bromo residue in the helix resulted in a 22° elevation of Tm. In the mono G-poly C complexes reported above a similar elevation of Tm for the G:BC pairs was observed. Experiments designed to aid our understanding of the reason for this effect of the 5-bromo residue are in progress.

Poly 8-aminoadenylic acid has been synthesized and a preliminary investigation made of its interaction with poly U. The new polymer has the interesting potential of forming three hydrogen bonds to the uridine residues of the third (Hoogsteen or reversed Hoogsteen) strand. It is clear that poly 8-AA can form a three-stranded and very probably a two-stranded helix with poly U. The details of stoichiometry and conditions governing relative stabilities of the two- and three-stranded complexes remain to be determined.

Infrared studies of t-RNA from several biological sources show extensive specific base pairing in these molecules.

In work with Drs. Orgel, Sulston, and Lohrman, of the Salk Institute, we have demonstrated nonenzymatic synthesis of oligoadenylates on a polyuridylic acid template. The reaction is specific with respect to base pairing potential of both template and monomer and occurs only under conditions of helix formation between monomer and polymer. The stereochemistry of the principal product is 2'-5', and the condensing agent is a water-soluble carbodiimide.

In NMR work on cytosine derivatives with Dr. R. Shoup and Dr. E. Becker (LPB) we have found rotational barriers in several neutral molecules as well as in the previously studied cytosine cations. In at least some cases the barrier is solvent-dependent and may be due to hydrogen bonding at an electronegative atom (N_3 or C_2O) remote from the bond in question. Further monomer-monomer base pairing studies in organic solvents have been carried out.

Publications:

Shoup, R. R., Miles, H. T., and Becker, E. D.: Steric control of geometrical isomerism in cytosine cations. A nuclear magnetic resonance study. J. Am. Chem. Soc. 89: 6200-6204, Nov. 1967.

Sulston, J., Lohrman, R., Orgel, L., and Miles, H. T.: Nonenzymatic synthesis of oligoadenylates on a polyuridylic acid template. Proc. Nat. Acad. Sci. 59: 726-733, March 1968.

Serial No. NIAMD-LMB- 8
1. Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biochemical control mechanisms in histidine biosynthesis

Previous Serial Number: NIAMD-LMB-11

Principal Investigator: Robert G. Martin

Other Investigators: Dr. Michal Bagdasarian (12/12), Dr. David Gutnick (7/12), Dr. Harvey J. Whitfield, Jr. (12/12), Dr. Bruce Ames (6/12).

Cooperating Units: Dr. P. Hartman, Johns Hopkins University, Baltimore, Md., Dr. N. Talal, Arthritis & Rheumatism Branch, NIAMD, Dr. David Berkowitz, University of Pennsylvania Medical School, Dr. B. N. Ames, University of California, Berkeley, Dr. G. Fink, Cornell University.

Man Years:

Total:	6
Professional:	4
Other:	2

Project Description:

Objectives: The pathway of histidine biosynthesis in S. typhimurium involves 10 enzymes. The genes for these enzymes are clustered in a segment of the Salmonella chromosome. The mechanism(s) that regulate the rate of synthesis of these enzymes are being investigated to try and understand the phenomenon of repression and coordinate enzyme synthesis.

Major Findings: Polarity (G. R. Fink and R. G. Martin). 1) a) Polar mutants have been examined in three additional genes of the histidine operon and it has been found that steep gradients of polarity are observed only in the first gene. This observation suggests that the extent of polarity generated by a nonsense mutant is a much more complicated function than previously realized. One possible explanation of this phenomenon depended upon the hypothesis that polarity in the first gene of an operon involved an interaction between mRNA translation and transcription. Experiments are currently in process to attempt to examine this directly.

b) (R. G. Martin). It is currently accepted that mutations in the relaxed RNA control locus disrupt the interaction between protein synthesis and mRNA production. If the basis for this interaction involved the DNA dependent RNA polymerase, and if polarity also involved this enzyme, then alterations of polarity would be predicted in relaxed strains of S. typhimurium. A relaxed RNA control mutant was isolated by a new technique involving radioisotope uptake under conditions of amino acid starvation and the appropriate polar, relaxed mutants were constructed. No affect of the relaxed locus on polarity was observed, demonstrating the error of one of the two hypotheses.

c) (R. G. Martin & N. Talal). Extensive genetic mapping of the polar hisC mutants demonstrated as previously suggested that the extent of polarity is clearly a function of some parameter in addition to the distance between the mutations and the subsequent gene start.

2) (D. Berkowitz, J. Hushon, H. J. Whitfield, Jr., J. Roth and B. N. Ames). A rapid procedure for the identification of UAG, UAA and UGA mutations has been devised. It is based on the ability of such mutations to revert consequent to a suppressor mutation. The suppressor mutation is identified by its ability to allow growth on lactose when the bacterium receives a F' lac episome carrying a UAG, UAA or UGA mutation.

3) (D. Gutnick, B. N. Ames and R. G. Martin). One of the difficulties in carrying out genetic mapping of the histidine region has been the lack of closely linked outside genetic loci. Aro E mutants are blocked in the synthesis of shikimic acid. Shikimic acid cannot be substituted for the end product amino acids due to the inability of the bacterium to transport this intermediate. Secondary mutants of S. typhimurium have been isolated in a locus designated aro D that can now grow on shikimic acid. This locus is closely linked by transduction with the histidine operon. The exact orientation of this locus is currently being established.

Publications:

Fink, G., Klopotowski, T. and Ames, B. N.: Histidine regulatory mutants in Salmonella typhimurium. IV. A positive selection for polar histidine-requiring mutants from histidine operator constitutive mutants. J. Mol. Biol. 30, 81-95, Dec. 1967.

Fink, G. and Martin, R.: Polarity in the histidine operon. II. J. Mol. Biol. 30, 97-107, Dec. 1967.

Voll, Mary Jane: Polarity in the histidine operon. III. The isolation of prototrophic polar mutations. J. Mol. Biol. 30, 109-124, Dec. 1967.

Martin, R. G.: Polarity in relaxed strains of S. typhimurium. J. Mol. Biol. 31, 127-134, Feb. 1968.

Serial No. NIAMD-LMB-9
1. Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interactions among replicons in E. coli: the bacterial genome, the fertility factor, and a temperate bacteriophage.

Previous Serial Number: NIAMD-LMB-12

Principal Investigator: Michael Yarmolinsky

Other Investigators: Maxwell Gottesman, Judah L. Rosner

Cooperating Units: Dr. David Korn, Laboratory of Biochemical Pharmacology, NIAMD (Project No. 384-74001-41); Dr. Robert Weisberg, Oak Ridge National Laboratories, Oak Ridge, Tennessee; Dr. René Thomas & Mme Suzanne de Leener-Mousset, Université Libre de Bruxelles, Belgium.

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives: An understanding, eventually in molecular terms, of regulatory mechanisms which control DNA replication and specific rearrangements in DNA structure due to recombination. Specifically our attention is focused on:

(a) The activation of one replicon by damage to another. (Indirect UV induction of bacteriophage lambda),

(b) The extent to which genetically autonomous replicons are associated. (Co-segregation patterns of F, bacterial chromosome, and a non-integrating λ),

(c) The reversible insertion of one replicon within another (lysogenization, prophage detachment, and curing),

(d) Organization of an international lysogeny workshop.

Each of the objectives mentioned above has been approached in separate but complementary studies.

Methods employed: Standard microbiological techniques and published modifications developed in this laboratory have been employed.

Major findings:

(a) The activation of one replicon by damage to another: parallel behavior of F and P1 in causing induction of lysogenic bacteria (Rosner and Yarmolinsky)

A variety of genetic elements (replicons) may coexist with the bacterial chromosome so as to be stably inherited. Two classes of replicons may be distinguished according to how coordination of replication with the bacterial chromosome is accomplished. Replicons of class I (e.g. lambdoid phages) accomplish this coordination by integration into the bacterial chromosome and are perpetuated as a set of bacterial genes. Replicons of class II (e.g., the sex factors) accomplish this coordination without integration. The means of coordination is unknown but may involve a cell-membrane element.

F, col I and RTF belong to class II. If these genetic elements are subjected to UV irradiation in one bacterium and subsequently transferred to a recipient harboring an inducible prophage, the lysogen is induced.

Bacteriophage P1 may belong to class II since, although it is stably inherited as prophage, there appears to be no chromosomal location for the P1 genome. We find that infection of λ lysogens with UV-irradiated P1 induces the λ prophage. There is no induction of λ$\underline{ind}^-$ prophage, by either irradiated P1 or irradiated F. An undamaged replicon appears dominant over an irradiated one. Thus, the presence of an F particle in the (F^- phenocopied) lysogenic recipient interferes with induction by an irradiated F. Similarly, P1 prophage in the recipient interferes with induction by irradiated P1.

The phages 434 and ϕ80 belong to class I. When irradiated and used to infect a λ lysogen, they cause no induction.

The irradiated replicons of class II may induce prophage by affecting the replication of the bacterial chromosome via the same system which normally insures their coordinate replication.

This work represents an extension of previous work by J. L. Rosner and Michael B. Yarmolinsky on induction by irradiated F. Since F particles (like the other sex factors, col I and RTF) do not occur as free extracellular particles, they are much more difficult to study than a bacteriophage. The finding that P1 may be representative of class II replicons is therefore of considerable interest.

(b) The extent to which genetically autonomous replicons are associated. This work, described in the previous project report, has been satisfactorily completed and published.

(c) Lysogenization, prophage detachment and curing

A. Studies in Lambda Lysogeny

I. Alternative modes of prophage integration and excision from *single lysogens*. (Gottesman & Yarmolinsky). The normal mode of prophage integration and excision requires a phage-determined Int function to promote site-specific recombination between prophage and bacterial genomes. However, defects in the normal integration system do not entirely eliminate the capacity for lysogenization. Homologies between the λ and bacterial genomes outside the usual prophage attachment site can be used for prophage integration at abnormal locations in bacteria which are *rec*$^+$. Lysogens carrying λ*int* at the normal location can be constructed by co-infection with an *int*$^+$ phage. The deletion mutant λb2, partially defective in the attachment region, lysogenizes inefficiently, but at the normal location. Relative to induction of λ^+, induction of single λ*int* or λb2 lysogens yields $<10^{-4}$ as many active phage but 20-60 times as many transducing particles. The aberrant excisions which generate transducing particles produce corresponding deletions recoverable in sub-lethally induced bacteria. The major product of λb2 or λb2*int* induction, λb2 *bio* uvrB, is unstable if *int*$^+$; with low efficiency it is freed of the bacterial component by Int function during vegetative growth.

II. *Prophage replication and excision of recombinants from double lysogens*. (Yarmolinsky & Gottesman, with collaboration specifically on Ter function, from Suzanne de Leener-Mousset and René Thomas). The homology afforded by a second prophage permits alternative modes of integration and excision. Thus, in the absence of Int function, bacterial Rec function permits integration of one prophage within another. Efficient prophage excision occurs among tandem double lysogens defective in as many as all three recombination functions: Int, Red and Rec. This may be due to release of a recombinant genome by a system (Ter) cutting at positions of vegetative phage termini. Where only one of two heteroimmune prophages in a tandem *int* lysogen is heat-inducible, thermal induction may yield a large burst of phage carrying the *un*induced immunity. This may be a consequence of an initial fusion of the inactive replicon with one which is active.

B. *Interaction of λdg with the bacterial chromosome*. (Weisberg and Gottesman) Lambda *dg* is defective in integration and excision due to a structural defect analogous to that of λ*b2*, rather than to the absence of a diffusible gene product. This conclusion is based upon the following observations:

1) When λ*dg* lysogenizes a cell with two attachment sites - one vacant and one occupied by a heteroimmune prophage - unlike λ^+, it attaches preferentially at the occupied site;

2) Prophage λ*dg* *c*I*857*, when temporarily derepressed by brief heat treatment, does not efficiently cure itself, but can, nevertheless, detach a structurally normal, heteroimmune prophage from an episomal site in the same cell. Efficient curing of λ*dg* occurs when the double lysogen is *cis*.

3) The promotion of λ*dg* integration by helper phages occurs only if the helper phage is itself integrated prior to the λ*dg*. The helper contributes a structural component required for efficient attachment of λ*dg* to the

bacterial chromosome.

The mode of λdg integration in the absence of helper phage, a project initiated by Gottesman, Korn and Yarmolinsky, has not been further pursued.

(d) Organization of Lysogeny Workshop, Sorrento, Italy, March 28-31.

Contributed with Drs. Amati, Calef, Gellert, Gottesman, Korn, Terzi and Weissbach to the organization of a meeting at which papers from this laboratory were presented on projects (c). This workshop represents the second international meeting devoted specifically to lysogeny. The first meeting was supported entirely by the NIAMD, NIH. Both meetings have been outstanding in the quality of the work presented and the impetus given to further developments in this expanding area of research.

Publications:

Rosner, J. L., Adelberg, Edward A. and Yarmolinsky, Michael B.: An upper limit on β-Galactosidase transfer in bacterial conjugation. J. of Bacter. 94, 1623-1628, Nov. 1967.

Gottesman, M. W. and Yarmolinsky, M. B.: Integration-negative mutants of bacteriophage lambda. J. Mol. Biol. 31, 487-505, Feb. 1968

Yarmolinsky, M. and Korn, D.: Evidence for independent segregation of the Escherichia coli chromosome and non-replicating bacteriophage λb2. J. Mol. Biol. 32, 475-479, Mar. 1968.

Serial No. NIAMD-LMB-10
1. Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Genetic studies of fused operons and transport deficient mutants

Previous Serial Number: None

Principal Investigator: Mark Levinthal

Other Investigators: None

Cooperating Units: Dr. P. E. Hartman, Dr. Robert Simoni, Dr. Saul Roseman, Johns Hopkins University, Baltimore, Md.; Dr. Hiroshi Nikaido, Mass. General Hospital, Boston, Massachusetts

Man Years:

Total:	4/12
Professional:	4/12
Other	0

Project Description:

Objectives: Deletion mutations fusing the histidine operon and the rfb operon have been obtained. Such deletions leave both operators intact. An increased rate of enzyme synthesis of either operon leads to a corresponding decrease of enzyme synthesis in the other operon. Current studies are in progress to determine the physiological basis of this interaction.

Mutants have been isolated which cannot utilize nine carbohydrates for growth. Genetic and biochemical studies are in progress to elucidate the nature of the lesion in sugar utilization and to map the function(s) with respect to known markers on the chromosome.

Major Findings: 1) Fused operons: The basis of inhibition appears to be a competition for segments of DNA by RNA polymerase molecules proceeding in different directions from each operator. The quantitative levels of recovery from inhibition by the histidine operon upon derepression are consistent with a simple competition model and experiments are in progress using nonsense mutants to prove that the competition is on the transcriptional level.

2) Carbohydrate mutants: These mutants have been divided into two classes. Each class results from a single gene event leading to a loss of a component of the phosphotransferase system. This enzyme system is responsible for the active transport of carbohydrates. Such mutants will not

actively transport carbohydrates but will carry out facilitated diffusion. The loss in ability to utilize these sugars for growth is caused by an inability to transport carbohydrates at sufficiently fast rate. One class maps near purC (order purC, carB, aroD, his). The other class, carA, maps between thr and pro. Experiments are in progress to isolate mutants which cannot carry out facilitated diffusion, in the hope that such mutants will lack enzyme II of the phosphotransferase system.

Publications:

Nikaido, H., Levinthal, M., Nikaido, K., and Nakane, K.: Extended deletions in the histidine - rough B region of the *Salmonella* chromosome *Proc. Natl. Acad. Sci.*, 57: 1825-1832, June 1967.

Simoni, R., Levinthal, M., Kundig, F. Dodyk, Kundig, W., Anderson, B., Hartman, P. E. and Roseman, S.: Genetic evidence for the role of a bacterial phosphotransferase system in sugar transport. *Proc. Natl. Acad. Sci.*, 58: 1963-1970, Nov. 1967.

Schiff, J. A. and Levinthal, M.: Studies of sulfate utilization by algae 4. Properties of a cell-free sulfate reducing system from *Chlorella Plant Physiol.*, in press, 1968.

Levinthal, M. and Schiff, J.: Studies of sulfate utilization by algae 5. Identification of thiosulfate as a major acid-volatile product formed by a cell-free sulfate reducing system from *Chlorella Plant Physiol.*, in press, 1968.

Hodson, R., Schiff, J., Scarsella, A. and Levinthal, M.: Studies of sulfate utilization by algae 6. Adenosine-3'-phosphate-5' phosphosulfate (PAPS) as an intermediate in thiosulfate formation from sulfate by cell-free extracts of *Chlorella Plant Physiol.*, in press, 1968.

Serial No. NIAMD-LMB-11
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: X-ray Diffraction Analysis of Protein Structure

Previous Serial Number: NIAMD-LMB-13

Principal Investigator: David R. Davies
Gerson Cohen
Brian Matthews
Enid Silverton

Other Investigator: Hazel Braxton

Cooperating Units: None

Man Years
Total: 5-1/2
Professional: 4
Other: 1-1/2

Project Description:

Objectives: The determination of the 3-dimensional structure of γ-chymotrypsin.

Methods Employed: X-ray diffraction analysis of crystals of gamma chymotrypsin by the heavy atom isomorphous substitution method.

Major Findings: A 5.5 Å electron density map of tosyl-γ-CHT has been computed based on the following heavy atom derivatives: pipsyl-γ-CHT, p-methoxy-m-mercuribenzene sulphonyl-CHT, mercuriiodide, and iodination of the enzyme crystals. This map shows no unusual properties: e.g., it has no high electron density on the symmetry axes. In addition a surface may be defined for the molecule, and all the heavy atom derivatives lie close to this surface. One feature of the map, a long rod of high density has been identified as corresponding to the only stretch of α-helix observed in α-CHT.

Simultaneously, a model of α-CHT has been constructed based on the 2.0 Å resolution electron density map of Matthews, Sigler, Henderson & Blow, 1967, and coordinates have been measured for each of the 1,500 atoms in the molecule. We have used a number of heavy atom sites common to both molecules in order to compare the molecules of α- and γ-CHT. This comparison enabled us to confirm the α-helix assignment and led to a transformation of the γ-CHT molecule into the unit cell of α-CHT based on minimizing the root mean square discrepancy between the two sets of heavy atom positions. The final value of this discrepancy was 1.6 Å, thus indicating that the two molecules are very similar within the limits of 5.5 Å resolution and relatively few sites.

Two differences can be observed between α-CHT and γ-CHT. The first has to do with the iodination of the molecule: chymotrypsin in solution (Glazer & Sanger, 1964) is iodinated preferentially on tyrosine 146. In the crystals of α-CHT, however Tyr 171 is preferentially iodinated, since Tyr 146 is buried in the surface of an adjacent molecule and is inaccessible to iodination. In γ-CHT the packing arrangement is different and as a result it is possible to iodinate both Tyr 146 and Tyr 171. Examination of the positions of the two iodines attached to Tyr 146 show that it occupies slightly different positions in the two molecules, being shifted by a translation of 3 Å and a rotation of about 60°.

The second difference involves the changes that accompany tosylation of the molecule. The most striking feature observed in a difference synthesis of (tosyl-γ-CHT) - (γ-CHT), apart from a peak identified as the sulfonyl of the tosyl group, is a large hole, roughly comparable in size to the sulfonyl peak. This hole is situated at the active site region of the molecule, about 5 Å from histidine 57, and we have identified it as being due to the displacement of a sulfate ion on tosylation of the molecule. This feature does not occur in comparable maps of α-CHT where it appears that it would be too close to the hydroxyl group of Tyr 146 of a neighboring molecule.

Significance to Bio-medical Research and the Program of the Institute: A knowledge of the 3-dimensional structure of proteins is an essential prerequisite for an understanding of the molecular mechanism of action of enzymes.

Proposed Course of Project: We are at present in the middle of collecting 2.7 Å data for the γ-CHT crystals, and we hope that, within the next few months we shall be able to compute a map at this resolution in order to make a more detailed comparison between α-CHT and γ-CHT. Since the major observed differences between the two molecules involve the active site region, it is intended to carry out an investigation of the binding of inhibitors to the molecule in order to obtain information about the mechanism of action. In addition, we are engaged in a search for new crystalline proteins for future structural investigations.

Publications:

Davies, D. R.: Symmetry of Helical Structures. J. Chimie Physique *65*: 15-22, Jan. 1968.

Matthews, B. W., Cohen, G. H., Silverton, E. W., Braxton, H., and Davies, D. R.: The Relation between γ- and α-Chymotrypsin. J. Mol. Biol. 1968.

Matthews, B. W.: Some crystal forms of bovine chymotrypsinogen B and chymotrypsinogen A. J. Mol. Biol. 33: 1968.

Matthews, B. W.: Solvent content of protein crystals. J. Mol. Biol. 33: 1968.

Serial No. NIAMD-LMB-12
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Enzymatic Joining of DNA Strands

Previous Serial Number: NIAMD-LMB-14

Principal Investigators: Martin Gellert
Steven B. Zimmerman

Other Investigators: John W. Little
Carol K. Oshinsky

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives: To understand the mechanism of action and physiological role of the DNA ligase of *E. coli*.

Methods Employed: Ultracentrifugation, standard enzymological techniques.

Major Findings: DNA ligase catalyzes the covalent closure of single-strand breaks (nicks) in duplex DNA. The reaction requires 5'-phosphoryl and 3'-hydroxyl termini on the apposed DNA strands. The enzyme from *E. coli* uses DPN as cofactor; for each new phosphodiester bond made, one molecule of DPN is cleaved to adenylate (AMP) and nicotinamide mononucleotide (NMN).

In further studies of the reaction mechanism, we have shown that the ligase catalyzes an exchange of NMN into DPN in the absence of DNA. This suggested an enzyme-adenylate intermediate, which we have since isolated: after DNA ligase is incubated with DPN, an enzyme-adenylate complex can be separated by filtration through Sephadex G-25. This complex is capable of sealing single-strand breaks in DNA (in the absence of DPN), liberating AMP. Our present understanding of the reaction can thus be summarized as follows:

1) DPN + ligase ⇄ ligase-AMP + NMN

2) Nicked DNA + ligase-AMP → sealed DNA + ligase + AMP

Nicked DNA + DPN → sealed DNA + AMP + NMN

Attempts to find a DNA-bound intermediate have not yet been successful, but are continuing.

Our efforts to understand the physiological role of DNA ligase have concentrated on searching for *E. coli* mutants with a defective ligase. Screening of a large number of *E. coli* strains with defects in repair of radiation damage, recombination, or DNA replication has so far not revealed any strains with an altered ligase, but such efforts are being continued.

Significance to Bio-medical Research and the Program of the Institute: This work is designed to gain a biochemical understanding of intracellular DNA repair and genetic recombination.

Proposed Course of Project: Further studies on the reaction mechanism are planned, as well as an extension of the mutant screening project.

Publications:

Zimmerman, S. B., Little, J. W., Oshinsky, C. K., and Gellert, M.: Enzymatic joining of DNA strands: A novel reaction of diphosphopyridine nucleotide. *Proc. Nat. Acad. Sci.* 57: 1841-1848, June 1967.

Little, J. W., Zimmerman, S. B., Oshinsky, C. K., and Gellert, M.: Enzymatic joining of DNA strands, II. An enzyme-adenylate intermediate in the DPN-dependent DNA ligase reaction. *Proc. Nat. Acad. Sci.* 58: 2004-2011, Nov. 1967.

Serial No. NIAMD-LMB-13
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interaction of DNA with Basic Polypeptides and Histones.

Previous Serial Number: NIAMD-LMB-15

Principal Investigator: Gary Felsenfeld

Other Investigators: J. T. Shapiro (Guest Worker)
F. Bernardi
B. J. Sebring

Cooperating Units: None

Man Years
Total: 4
Professional: 2-1/2
Other: 1-1/2

Project Description:

Objectives: The objective of this project is to study the interaction of basic polypeptides and histones with DNA, in order to determine the mechanism and biological significance of the base-composition specific interactions previously observed in this laboratory.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound.

Major Findings: We have continued to investigate the physical basis of the selectivity of polylysine for adenine-thymine (A-T) rich DNA. We find that the interaction of DNA with polylysine results in the formation of micelles of remarkably uniform size. Light scattering and sedimentation velocity measurements are consistent with the presence of highly hydrated spherical particles with an average radius of 1700 Å. The optical rotatory dispersion of these micelles is quite different from the ORD of DNA in solution. There is a large trough at 2800 Å, for example, which is an order of magnitude greater in size than the peak observed for DNA alone in this region of the spectrum. Since all obvious artifacts have been eliminated as possibilities, we conclude that either the polylysine-DNA complex involves a very close approach of a perturbing chromophore of the polypeptide to the DNA, or that the structure of the DNA itself is altered in the complex (for example, by tilting of the bases). We have found that poly-L-lysine and poly-D-lysine exhibit similar behavior, but that the random copolymer poly-DL-lysine binds to DNA much more weakly. This suggests that the poly L (or D) lysine complex with DNA involves a regularly ordered structure, rather than a non-specific interaction of the kind often observed between polyanions and polycations.

In order to determine the nature of the specificity of polylysine for A-T pairs, we have examined the interaction of DNA with a variety of small molecules. It has been found in our laboratory that the selectivity of polylysine for A-T rich DNA is reversed by tetramethylammonium ion (TMA^+), so that G-C rich DNA is selected instead. Binding competition studies using dialysis equilibrium show that although sodium, lithium, potassium or cesium ions are bound equally to DNA's of varying base composition, TMA^+ is preferentially bound to A-T rich DNA. We find that a series of DNA's of increasing A-T content bind TMA^+ with increasing affinity. The reversal of selectivity of polylysine by TMA^+ is thus the result of the competition for A-T rich DNA offered by TMA^+.

Our binding experiments also show that tetraethylammonium ion is selective for A-T pairs, while the larger tetrabutylammonium ion is not. These results suggest that TMA^+ is bound preferentially to individual A-T sites, and it is not unreasonable to suppose that the selective mechanism in the case of polylysine is similar.

We are extending our studies of nucleic acid-polypeptide interactions to the investigation of nucleohistone complexes. We have improved existing methods for the fractionation of histones, using techniques that do not expose the proteins to extreme conditions.

Significance to Bio-Medical Research and the Program of the Institute: Biologically important polycations, particularly the histones, are suspected of having a role in the regulation of DNA-primed RNA synthesis in higher organisms. Such regulatory properties would presumably require that there exist a mechanism for specific interaction between the regulatory molecule and specific sites on DNA. Understanding of regulatory mechanisms is one of the principal goals of biological research. It appears possible that the selectivity of polylysine and polyarginine may be related to a similar (though more complicated) selectivity of naturally occurring histones for certain sequences of DNA bases in nucleohistone complexes.

Proposed Course of Project: Further chemical studies are being carried out of the processes by which various small molecules distinguish between A-T and G-C pairs. It is our hope the histone fractionation methods will permit direct study of possible selective reactions between histones and DNA and suitable experiments are being planned.

Serial No. NIAMD-LMB-14
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Structure of Single Strand Polynucleotides in Solution.

Previous Serial Number: NIAMD-LMB-17

Principal Investigator: Gary Felsenfeld

Other Investigator: L. D. Inners (Guest Worker)

Cooperating Units: None

Man Years
Total: 2
Professional: 1-1/2
Other: 1/2

Project Description:

Objectives: To elucidate the structure of single strand polyribonucleotides in solution.

Methods Employed: Theoretical analysis, light scattering, viscometry, spectrophotometry, sedimentation velocity, sedimentation equilibrium.

Major Findings: We have extended the measurements carried out on polyriboadenylic acid (poly A) to another single strand molecule, polyribouridylic acid (poly U). We find that poly U can be separated into fractions of well defined molecular weight by phase separation methods closely related to those used for poly A. We are able to find ideal solvent conditions for poly U. The conditions are quite close to those previously found for poly A. This is particularly fortunate because it makes possible a direct comparison of the properties of poly A, which forms a base stacked structure, and of poly U, which probably does not. We commenced calculations of the expected dimensions of polymer coils with known restrictions placed upon bond angles and freedom of rotation, in order to compare theory with the experimental results. In the course of this work extensive use has been made of short column equilibrium sedimentation for the determination of molecular weights. Using the scanning photomultiplier accessory of the ultracentrifuge, existing techniques have been refined to the point of yielding weight average molecular weights which agree within 2% with those obtained by light scattering, but require less than a microgram of material for the determination.

Significance to Bio-medical Research and the Program of the Institute: Poly A and poly U structure may bear a resemblance to structures which could occur in natural RNA's which are not able, because of their base composition, to form satisfactory hydrogen bonds. The structures of various kinds of RNA in solution are intimately connected with their role in protein synthesis. This project represents the first analysis of a polynucleotide using the methods of classical polymer chemistry in a rigorous manner. Availability of data of the kind obtained makes possible thermodynamic and other theoretical analysis not possible heretofore.

Proposed Course of Project: Studies of poly U and other polynucleotides will be continued, and detailed calculations of polynucleotide dimensions from "first principles" will be carried out. Because of the use of ideal conditions, results of such calculations can be compared directly with our experiments.

Publications:

Eisenberg, H., and Felsenfeld, G.: Studies of the temperature-dependent conformation and phase separation of polyriboadenylic acid solutions at neutral pH. J. Mol. Biol. 30: 17-37, Nov. 1967

Hirschman, S. Z., Gellert, M., Falkow, S., and Felsenfeld, G.: Spectral Analysis of the Intramolecular Heterogeneity of λ DNA. J. Mol. Biol. 28: 469-477, Sept. 1967.

Serial No. NIAMD-LMB-15
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Calorimetric Studies of Nucleic Acid Interactions

Previous Serial Number: NIAMD-LMB-18

Principal Investigator: Philip D. Ross

Other Investigator: Robert L. Scruggs

Cooperating Unit: Mr. Thomas Clem, DRS-BEI

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives: To evaluate the energetic contributions of the various chemical components of nucleic acids; sugars, phosphate groups, hydrogen bonds, stacking interactions, to the thermodynamic stability of nucleic acid helical complexes.

Methods Employed: Calorimetry

Major Findings: A differential microcalorimeter for the study of thermally induced processes in solution has been constructed. It is possible with this apparatus to heat a small sample of solution (2.5 ml) from 10° to 80° and measure a heat effect occurring over a 20° temperature range and amounting to 20 cal/litre with a precision of 5%. Calibration experiments indicate this equipment yields results in excellent agreement with those of other investigators. Application of this technique to the study of phase transformations will yield both the energy and entropy of these transitions. The very general nature of this equipment makes it applicable to the study of many other types of material, e.g., denaturation of proteins, and to the study of thermally induced chemical and biological processes.

Significance to Bio-medical Research and the Program of the Institute: Use of this apparatus will provide fundamental thermodynamic information on helix-coil transformations in nucleic acids.

Proposed Course of Project: This work is being continued.

Annual Report of the

MATHEMATICAL RESEARCH BRANCH

NIAMD

Individual research projects, collaborative research and consultation with scientists in the various Institutes and Divisions have continued substantially along the general lines and within the general areas indicated in the summary for last year. Highlights of completed work and newly initiated research are summarized below. A general survey will show that the results are a considerable mix of pay-off in applications from previous theoretical studies and new or extended theoretical and mathematical investigations.

Mathematical and computational studies relevant to experimental neurophysiology have been continued. Time and effort have been divided between (a) exploration of further implications and extensions of theoretical solutions for extracellular potential distributions generated by activity in dendritic neurons, (b) communication of the results of a collaboration with Drs. Frank, Nelson, Burke and Smith (NINDB) which demonstrates substantial agreement between experimental observations of various excitatory post-synaptic potentials from cat spinal motoneurons and the predictions computed with a theoretical model providing for various dendritic and somatic distributions of synaptic input, and (c) communication of the results of a theoretical study with Dr. G. Shepherd (Yale University) of olfactory bulb field potentials which led to a prediction of two-way dendro-dendritic synaptic interactions which prediction has received strong support from the independent, electron-micrographic studies of Drs. Reese and Brightman (NINDB). (Dr. W. Rall)

Work on the extended model for transport, diffusion and chemical reaction of substances in the blood-capillary-tissue complex has been continued with special reference to oxygen consumption in muscle. The mathematical formulation, numerical methods, computer program and theoretical discussion with implications of the model have been completed for the steady-state case. A paper setting forth these results has been accepted for publication in Mathematical Biosciences. The more difficult analysis of transient responses is well under way. (Dr. J. Gonzalez-Fernandez and Mrs. S. Atta)

Research on the kinetics of an enzyme-substrate-modifier system has been continued and extended to the case in which a modifier-substrate complex is the active substrate. This case can be kinetically distinguished from that in which the modifier activates the enzyme as well as forms the active substrate. In both cases methods for determining all of the kinetic constants of the system from steady-state rate data have been devised. (Dr. W. London)

Last year a successful computer program for approximating data by positively weighted exponentials was reported. The efficiency and usefulness of this program has been greatly improved by the addition of an explicit method, based on symmetric differences, for dealing with singularities which arise in the course of the computation. The spectral properties of the matrix resulting from the linearization, about a stationary point, of a class of axon equations have been studied. Some properties previously demonstrated by Cole et al for the Hodgkin-Huxley axon have been generalized. (Dr. J. Evans)

A mathematical model describing the kinetics of glucose oxidation to CO_2 via the Embden-Meyerhof-TCA path (I) and the hexosemonophosphate shunt (II) has been developed. Analysis of data from human subjects show that hypothyroidism is associated with decreased rate of irreversible loss of plasma glucose and a decreased fraction of glucose oxidized via I, while the fraction oxidized via II is unchanged. Hyperthyroidism is associated with normal rate of irreversible loss and fraction oxidized via I, while the fraction oxidized via II is markedly reduced. (Dr. D. Shames with Drs. Berman and Segal)

The development of modeling techniques has included methods for dealing with multiple studies and nonlinear control. In the study of calcium metabolism in man a series of dietary perturbations in both normals and abnormals were explained by the previously derived model. From these, the model was extended to include some regulatory pathways. (Dr. M. Berman with Drs. J. Phang and A. Kales, NCI) The calcium model for man was also applied successfully to the study of calcium metabolism in pregnant and lactating cows. (Dr. M. Berman with Dr. C. Ramberg, Univ. of Pennsylvania) Analysis and modeling of free fatty acids conversion to triglycerides in man has continued. The models tested provided a better understanding of the processes involved and also brought out some inconsistencies and shortcomings of the experimental procedures. (Dr. M. Berman with Drs. A. Frank and D. Steinberg, MHI) Thyroid studies of the Greek population were extended to include separate T_3 and T_4 studies and control individuals from non-endemic areas. These studies do not show major differences from similar studies in the States. (Dr. M. Berman with Dr. Koutras, Univ. of Athens) A unified approach for dosimetry calculations has been developed. This approach was adopted by the MIRD Committee of the Society of Nuclear Medicine as a schema for dose calculations. Computer program for dose calculations completed. (Dr. M. Berman with Dr. Loevinger, International Atomic Energy Agency, and Dr. J. Del Priori, DCRT)

Studies of properties of the diffusion-reaction equation have been extended with special reference to the connections between conditions of flux-equilibrium, chemical equilibrium and stationarity. These properties have been applied to the equations for tracer and specific activity in a spatially distributed open system. It has been shown that the washout curve for an arbitrary stationary compartmental system is strictly convex. (Dr. J. Hearon) The washout curve may be logarithmically concave or convex. However for a passive system it is always logarithmically convex. (Dr. J. Hearon with Drs. R. Gunn and C. Patlak) A set of continuous maps has been constructed each of which maps a class of generalized inverse onto a stronger class the elements of which are the fixed points of the map. These maps have been utilized in a study of differentiable generalized inverses and the classical relation between the derivative of a matrix and that of its inverse has been generalized. (Dr. J. Hearon with Dr. J. Evans)

Further studies of system connectivity and matrix properties were conducted. A compartmental system is said to be cuttable if deletion of one compartment disconnects the system, and cut reducible if deletion of one compartment makes flow from one subsystem to one or more other subsystems uni-directional. The matrices of such systems are characterized by possessing a diagonal element of which the cofactor is block diagonal or reducible, respectively. It was shown that such matrices enjoy properties analogous to those of block diagonal and reducible matrices and that use may be made of these properties in simplifying solutions of appropriate compartmental systems. (Mrs. R. Marimont, Associate Member, MRB)

Work continued on the theory of salt concentration in the kidney. The most important result was that a simple probablistic model of the steady state counter current multiplier was extended to the non-steady state. This gave an analytic solution for the rate of salt buildup in a hypothetical system in which salt is actively pumped from the ascending to the descending limb with no back leak. In the actual nephron there is almost certainly diffusion from the descending to the ascending link--a much more complicated problem. However the results for the simpler system illustrate many of the novel (and weird) features of systems of this kind. (Dr. J. Stephenson, Associate Member, NRB)

Serial No. NIAMD, ODIR, MRB-1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project: Mathematics of kinetics and reaction-transport system.

Previous Serial Number: NIAMD, ODIR, MRB-1

Principal Investigator: John Z. Hearon

Man Years:		
	Total:	1 1/2
	Professional:	1
	Other:	1/2

Project Description:

Objectives: The objectives of this research remain as stated for calendar year 1962.

Results and Significance: In addition to miscellaneous problems involving consultation and minor collaborative efforts, the work of the past year involved (1) further study of the diffusion-reaction equation (2) compartmental analysis and (3) matrix theorems and linear algebra.

Properties of the Diffusion-Reaction Equation.

In an extension of previous work (Bull. Math. Biophys. 27: 291-303, 1965) two theorems regarding the equation of continuity for an arbitrary open chemical system have been proved. One gives boundary conditions under which the flux vector for a given species can be guaranteed to be directed into the reaction region. The other shows that any two of the following conditions imply the third (i) flux equilibrium (ii) chemical equilibrium and (iii) stationarity. Applications of these results to active transport and to the next listed problem are given.

Equations for Specific Activity in Distributed Tracer System.

Except for certain approaches to circulatory mixing, the non-compartmental analysis of tracer systems is little developed. The derivation of the relevant differential equations was reported in the annual report of 1966. Further analysis has given the conditions under which (as always is the case under the compartmental assumption) the forms of the tag equations and specific activity equations are identical.

Washout Curve in Tracer Kinetics.

It has been shown that the washout curve for any stationary compartmental system is strictly convex for all $t > 0$. The second derivative may vanish at $t = 0$ and necessary and sufficient conditions are given for this. This last result shows that the log-plot of the washout curve may be concave.

In a collaborative study with Drs. R. Gunn and C. Patlak (NIMH) it has been shown by actual example that the log-plot may be convex, concave then convex or convex then concave then convex. However for a passive system, the log plot is strictly convex.

Spaces and Maps of Generalized Inverses.

In a study with Dr. John W. Evans, of this Branch, a collection of continuous maps is given each of which maps a class of generalized inverses onto a stronger class and the elements of the stronger class are fixed points of the map. These maps have been employed in the next listed problem.

Differentiable Generalized Inverses.

In a study (begun and reported last year) with Dr. John W. Evans, necessary and sufficient conditions are given for a differentiable matrix to have a differentiable generalized inverse. When these conditions are met there exists, for several classes of inverses, a differentiable inverse which coincides on a prescribed subset with a predetermined inverse. The classical relation between the derivative of a nonsingular matrix and its inverse is generalized to the singular case.

Generalized Inverses and Solutions of Linear Systems.

It is well known that if $Ax = y$ then A^+y is the least-squares solution of minimum norm where A^+ is the Moore-Penrose inverse. It is shown that a weaker class of inverse has the preperty that By is the solution of minimum norm if the system is consistent if B is in that class. Another class is shown to have the property that By is a least-squares solution. These results are used to show clearly the role played by the various classes of inverses in estimation problems in particular best linear unbiased estimates of estimable linear functions.

Publications:

Hearon, J. Z.: Construction of EPr generalized inverses by inversion of nonsingular matrices. J. RESEARCH, NBS 71B: (Math. and Math. Phys.) Nos. 2 and 3, 57-60, April-Sept. 1967.

Hearon, J. Z.: A generalized matrix version of Rennie's inequality. J. RESEARCH, NBS 71B: (Math. and Math. Phys.) Nos. 2 and 3, 61-64, April-Sept. 1967.

Hearon, J. Z.: Polar factorization of a matrix. J. RESEARCH, NBS. 71B: (Math. and Math. Phys.) Nos. 2 and 3, 65-67, April-Sept. 1967.

Hearon, J. Z.: Partial isometric matrices. J. RESEARCH, NBS, 71B: No. 4, 225-228, Oct-Dec. 1967.

Hearon, J. Z.: Symmetrizable generalized inverses of symmetrizable matrices. J. RESEARCH, NBS, 71B: No. 4, 229-231, Oct.-Dec. 1967.

Hearon, J. Z.: On the singularity of a certain bordered matrix. *SIAM J. Appl. Math.*, 15: 14-13-1421, Nov. 1967.

Hearon, J. Z.: Idempotent matrices with nilpotent difference, *Math. Mag*. 41: 80-83, Mar. 1968.

Hearon, J. Z. and Evans, J. W.: On spaces and maps of generalized inverses. *J. RES. NBS*, 72B, 1968.

Hearon, J. Z. and Evans, J. W.: Differentiable generalized inverses. *J. RES. NBS*, 72B, 1968.

Hearon, J. Z.: The washout curve in tracer kinetics. To appear *Mathematical Biosciences*, 1968

Hearon, J. Z.: The differential equations for specific activities in a distributed tracer system. *Bull. Math. Biophysics*, 30, No. 3, Sept. 1968.

Hearon, J. Z.: Properties of solution of the diffusion-reaction equation. II. *Bull. Math. Biophysics*, 30, No. 3, Sept. 1968.

Gunn, R. B., Patlak, C. S. and Hearon, J. Z.: The logarithmic convexity of the washout function in tracer kinetics. To appear *Math. Biosci.* 1968.

Serial No. NIAMD, ODIR, MRB-2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mathematical formulation and analysis of problems relevant to experimental neurophysiology.

Previous Serial Number: NIAMD, ODIR, MRB-2

Principal Investigator: Wilfrid Rall

Man Years:		
	Total:	1 1/4
	Professional:	1
	Other:	1/4

Project Description:

Over the past several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models. This group of models provides a theory that can account for many different sequences of events in a dendritic neuron, and in certain populations of such neurons. Computational experiments performed with these models provide theoretical predictions that can be compared with the results of neurophysiological experiments and neuroanatomical observations. Also, insights are obtained, which can lead to new hypotheses, new interpretations, and to the design of new experiments.

1. New theoretical explorations have been initiated. Dr. John Evans and I have been investigating extensions and further implications of my theoretical solution for the extracellular potential distribution generated by activity in a dendritic neuron, and of mathematical models for nerve membrane action potentials. Also, I have explored with Dr. Phillip Nelson (NINDB) the use of "equalizing time constants" to estimate the effective electrotonic length of motoneuron dendritic trees. These studies are still in progress and will be reported more fully next year.

2. Considerable time and effort were devoted to communication of research results, both by participation in several symposia and in the preparation and revision of manuscripts. Time and effort were given to referee theoretical manuscripts for several scientific journals.

3. With regard to dendritic synaptic activity in motoneurons of cat (described in last year's report) a series of five papers based upon a collaboration with Drs. Frank, Nelson, Burke and Smith (NINDB) has now been published in the Journal of Neurophysiology and has elicited considerable interest and comment (for example, Nature: 216, 1269, December 30, 1967).

Numerous informal communications from anatomists and physiologists now provide further observations that are interpreted as confirmatory of our theoretical predictions and interpretations. Briefly, we have demonstrated that a large variety of observations of synaptic potentials in motoneurons can be accounted for theoretically by means of various dendritic as well as somatic distributions of synaptic input.

4. The theoretical study of olfactory bulb field potentials (with Dr. Gordon Shepherd) has been described in previous reports. We have now completed a final revision of a comprehensive manuscript, and this has recently been submitted for publication. Our prediction of two-way dendrodendritic synaptic interactions and the strong support provided by the independent electron micrographic study by Drs. Reese and Brightman (NINDB), has been received with great interest at several symposia (for example, Science: 159, 335-353, Jan. 19, 1968).

Invited talks:

Lecture at the Scuola Normale Superiore (Pisa), sponsored by Professor Moruzzi of the Institute of Physiology, Pisa, Italy, July 1967.

Keele Symposium: Neural Communications--Experiment and Theory, sponsored by the International Brain Research Organization and the University of Keele, Staffordshire, England, September 1967.

Neurosciences Research Program, Work Session on Neural Coding, Brookline, Massachusetts, January 1968.

Physiology Society Symposium on New Developments in Vertebrate Synaptology, FASEB Meetings, Atlantic City, April 1968.

Biology Research Seminar, Hunter College, New York, City, May 10, 1968.

Publications:

Rall, W.: Distinguishing theoretical synaptic potentials computed for different soma-dendritic distributions of synaptic input. J. Neurophysiol. 30: 1138-1168, Sept. 1967.

Rall, W., Burke, R. E., Smith, T. G., Nelson, P. G. and Frank, K.: Dendritic location of synapses and possible mechanisms for the monsynaptic EPSP in motoneurons. J. Neurophysiol. 30: 1169-1193, Sept. 1967.

Rall, W.: Synaptic activity at dendritic locations: theory and experiment. In Caianiello, E. R. (Ed.): Proceedings on Neuronic Networks. Heidelberg, Germany, Springer-Verlag. (Galley proofs received in April, 1968.)

Serial No. NIAMD, ODIR, MRB-3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mathematical description of the transport-chemical reaction kinetics of substances in the blood-capillary-tissue complex.

Previous Serial Number: NIAMD, ODIR, MRB-3

Principal Investigator: Jose M. Gonzalez-Fernandez

Other Investigator: Susie E. Atta

Man Years: Total: 2 1/4
Professional: 2
Other: 1/4

Project Description:

Objectives: The objectives of this research remain substantially the same as those in the previous report.

Major results: The numerical method and its computer program as well as the theoretical discussion has been completed for the steady state case. A paper embodying these results has been accepted for publication.

Further work incorporating more complex geometrical properties of the capillary network has continued.

A study of the nature of the transient responses is under progress.

Publications:

Gonzalez-Fernandez, J. M. and Atta, S. E.: Transport and consumption of oxygen in capillary-tissue structures. To appear - Math. Biosci., 1968.

Serial No. NIAMD, ODIR, MRB 4.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Analysis of kinetic data and modeling.

Previous Serial No.: NIAMD, ODIR, MRB-4

Principal Investigator: Mones Berman

Other Investigators: Marjory F. Weiss, David Shames,
J. Phang (NCI), A. Frank and D. Steinberg (NHI)
and C. Romberg (Univ. of Pennsylvania)

Man Years:		
	Total:	3
	Professional:	3
	Other:	0

Project Description:

Objectives: The development of modeling techniques and their application to kinetic studies.

Major Findings: Methods for dealing with multiple studies and with nonlinear control have been investigated and applied.

In the study of calcium metabolism in man (Drs. J. Phang and A. Kales, NCI) a series of dietary perturbations in both normals and abnormals were explained by the previously derived model. From these, the model was extended to include some regulatory pathways. The findings also suggested new experiments that may shed new light on the metabolic pathways.

The calcium model for man was also applied successfully to the study of calcium metabolism in pregnant and lactating cows (Dr. C. Romberg, Univ. of Pennsylvania). From observations made and conclusions drawn by Dr. Romberg the model was further modified with possible extensions to man also.

Analysis and modeling of free fatty acids conversion to triglycerides in man has continued (Drs. A. Frank and D. Steinberg). The models tested provided a better understanding of the processes involved and also brought out some inconsistencies and shortcomings of the experimental procedures. Further testing of models and improvements in experimental techniques are expected to yield an improved and consistent model.

Other applications of data analysis and kinetics are discussed in accompanying reports. (Iodine Kinetics--Berman, Glucose--Shames)

Significance: These projects are very valuable not only in getting a better understanding of the systems studies but also in providing impetus for the development of new modeling techniques.

Proposed Course: The above areas of development will continue.

Invited Talks:

IVth International Biometrics Congress, Sidney, Australia.
1968 Duell Conference, Carmel, California

Publications:

Neer, R., Berman, M., Fisher, L. and Rosenberg, L. E.: Multicompartmental analysis of calcium kinetics in normal adult males. J. Clin. Invest. 46: 1364-1379, Aug. 1967.

Berlin, N. I., Berman, M., Berk, P. D., Phang, J. M. and Waldmann, T. A.: The application of multicompartmental analysis to problems of clinical medicine. Ann. Inter. Med. 68: 423-448, Feb. 1968.

Eaton, R. P., Berman, M. and Steinberg, D.: Kinetic studies of plasma free fatty acid and triglyceride metabolism in man. To appear J. of Clin. Invest., 1968.

Brownell, G. L., Berman, M., and Robertson, J.S.: Nomenclature for tracer kinetics. J.A.R.I. 1: 249-262, April 1968.

Serial No. NIAMD, ODIR, MRB 4.2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Iodine Kinetics in Man

Previous Serial No.: NIAMD, ODIR, MRB 4

Principal Investigator: Mones Berman

Collaboration Investigators: D. A. Koutras, University of Athens, Athens, Greece

Man Years:		
	Total:	.5
	Professional:	.25
	Other:	.25

Project Description:

Objective: To develop a model for the kinetics of iodine and thyroid hormones in man.

Major Findings: Studies of the Greek populations were extended to include separate T_3 and T_4 studies and control individuals from non-endemic areas. These studies do not show major differences from similar studies in the States.

Significance: This is part of an effort to test the derived model for iodine kinetics (published in JCE, 1968) to explain abnormalities, and the effects of radiation in the treatment of hyperthyroidism.

Future Course: Development of thyroid system will continue.

Publication

Berman, M., Hoff, E., Barandes, M, Becker, D. V., Sonenberg, M, Benua, R. and Koutras, D. A.: Iodine kinetics in man--a model. J. Clin. Endocr. and Metab. 28: 1-14, Jan. 1968.

Serial No. NIAMD, ODIR, MRB 4.3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: SAAM Computer System

Previous Serial No.: NIAMD, ODIR, MRB 4

Principal Investigator: Mones Berman

Other Investigators: Marjory F. Weiss

Man Years:	Total:	1 1/4
	Professional:	1 1/4
	Other:	0

Project Description:

Objectives: To develop a general purpose computer program for modeling bio-kinetic systems so that it may readily be used by investigators not sophisticated in mathematics or programming. The SAAM program was initially developed in 1959 and various phases of it have been in use ever since.

Major Findings: Because of its size and growth, development of SAAM could not be pursued on the NIH IBM 360 system. The operation was therefore switched to the NBS UNIVAC 1108 through a 1004 remote terminal. This turned out to be an excellent set-up, permitting us to integrate a large number of new features developed over the past few years (Mrs. Condliffe and Mrs. Weiss), and resulted in turn-around times of about 5 minutes. The cost of this operation is also very economical compared to the IBM 360/50 system.

Significance to Bio-medical Research and the Program of the Institute: SAAM is a general purpose program and permits easy modeling and solution of many diverse and complex problems. It is in routine use by this group and by others. Copies of the program have been supplied to many computer centers in this country and abroad.

Proposed Course: Continue the development of new features towards greater power, automation and routine use.

Publications:

Berman, M. and Weiss, M. F.: SAAM Manual. U. S. Dept. of Health, Education, and Welfare, Public Health Service Publ. No. 1703. Washington, D. C., U. S. Government Printing Office, 1967.

Serial No. NIAMD, ODIR, MRB-5
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Dosimetry from internally metabolized radionuclides

Previous Serial No.: NIAMD, ODIR, MRB-5

Principal Investigator: Mones Berman

Other Investigators: Robert Loevinger, International Atomic Energy Agency and James Del Priori, DCRT

Man Years:		
	Total:	1/2
	Professional:	1/2
	Other:	0

Project Description:

Objectives: Development of unified approach to the calculation of radiation dose from internally metabolized radionuclides and the development of a computer program for routine, automatic dose calculations.

Major Findings: Development of unified approach for dosimetry calculations completed. This approach was adopted by the MIRD Committee of the Society of Nuclear Medicine as a schema for dose calculations. Computer program for dose calculations (Del Priori) completed, although some fine points still need to be incorporated.

Future Course: It is expected that further development of the program will be sponsored by the MIRD committee. Collaboration will continue with MIRD.

Publications:

Loevinger, R. and Berman, M.: A schema for absorbed-dose calculations for biologically-distributed radionuclides. J. Nucl. Med., Suppl. No. 1, 7-14, Feb. 1968.

Loevinger, R., and Berman, M.: A formalism for calculation of absorbed dose from radionuclides. Phys. Med. Biol. 13: 205-217, Feb. 1968.

Serial No. NIAMD, ODIR, MRB-6
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mathematical Analysis of Biological Subsystems.

Previous Serial Number: NIAMD, ODIR, MRB-6

Principal Investigator: John W. Evans

Man Years: Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: To develop analytical procedures for use in understanding and in testing biological subsystems.

Major Activity and Results:

Approximation by Exponentials: a method (using symmetric differences) of explicitly handling the singularities that arise in computation has been added to the approximation routine described in last year's report.

Generalized Inverses: with Dr. J. Z. Hearon two papers on the properties of generalized inverses have been accepted for publication.

Linearized Axon Equations: spectral properties of the linearization of a class of axon equations at the stationary point have been examined and some properties demonstrated by Cole et al. in 1955 for the Hodgkin and Huxley axon have been generalized.

Electrophysiology: some implications and further development of the theoretical neuron models of Dr. W. Rall are being pursued with Dr. Rall.

Publications:

Evans, J. W., Cantor, D. G. and Norman, J. R.: The dead space in a compartmental lung model. Bull. of Math. Biophy. 29: 711-718, Dec. 1967.

Hearon, J. Z. and Evans, J. W.: On spaces and maps of generalized inverses. J. RES. NBS, 72B, 1968.

Hearon, J. Z. and Evans, J. W.: Differentiable generalized inverses. J. RES. NBS, 72B, 1968.

Serial No. NIAMD, ODIR, MRB-7
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Kinetic interactions of carbohydrate and lipid metabolism in man.

Previous Serial No.: None

Principal Investigator: David M. Shames

Other Investigators - Mones Berman
Stanton Segal, Children's Hospital
Philadelphia, Pa.

Man Years:	Total:	1.1
	Professional:	1.1
	Other:	0

Project Description:

Objectives: The objective of this research has been the development of a mathematical model describing the kinetics of glucose oxidation of CO_2 by the Embden-Meyerhof-tricarboxylic acid (EMP-TCA) and hexose-monophosphate (HMP) pathways in normal, hypo- and hyper-thyroid man at the basal steady state. This work has been completed and submitted for publication. The present objective is to incorporate this model into a larger model describing the interaction of glucose, insulin, free fatty acid and lipoprotein triglyceride in man. It is hoped that a model describing the interactions of these metabolites in normal man will aid in understanding the mechanism of some of the disorders of this system such as hyperlipidemia and its relation to glucose intolerance and insulin concentration.

Major Findings: Hypothyroidism is associated with a decrease in the rate of irreversible plasma glucose loss (ρ_{01}) and a decrease in the minimal fraction of glucose loss oxidized to CO_2 by way of the EMP-TCA (φ_t). The fraction of glucose loss oxidized to CO_2 by the HMP (Ψ) is unchanged from normal. The hyperthyroid studies suggest that ρ_{01} and φ_t are within the normal range. However Ψ is decreased to less than half the normal value.

ANNUAL REPORT OF CLINICAL INVESTIGATIONS, NIAMD

July 1, 1967 to June 30, 1968

Dr. Robert S. Gordon, Jr., Clinical Director

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During this fiscal year there were no major changes in the internal organization, or in the areas of program interest, within NIAMD Clinical Investigations. In addition to the Institute's permanent professional staff, there were 21 Clinical Associates, 4 Visiting Scientists, and 13 Guest Workers active in the clinical research areas. The latter two groups represented 7 countries in addition to the United States.

Honors or other recognition received by staff members during the year include:

> Election to membership in the Association of American Physicians: Dr. R. Gordon, Metabolic Diseases Branch,
>
> Election to the American Society for Clinical Investigation: Dr. N. Talal, Arthritis and Rheumatism Branch,
>
> Election to the American Chemical Society: Dr. P. Goldman, Arthritis and Rheumatism Branch.

Patient care statistics showed a slight decrease in overall activity since last year. From March 1, 1967 through February 29, 1968, 506 patients were admitted to the nursing units, for a total of 15,900 inpatient days. The average length of stay was 35 days, and the mean bed occupancy was 64%. During the same period, there were 2,409 outpatient visits for diagnosis or therapy in the clinics served by NIAMD physicians.

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ARTHRITIS AND RHEUMATISM BRANCH

The studies of the Arthritis and Rheumatism Branch include a range from purely molecular phenomena to the natural history of human disease.

I. Natural History of Disease

A series of 15 patients with primary amyloidosis has been rather thoroughly evaluated. Data has been assembled on the organs and tissues involved, on the metabolism of the various immunoglobulins, and on the peripheral circulatory responsiveness. A portion of the patients appeared to fall in a distinct category exhibiting anomalous serum proteins, macroglossia, carpal tunnel syndrome and amyloid purpura. (Drs. Barth, Willerson, Waldmann (C-MET) and Zelis (H-C)).

Another focus of interest was the granulocyte response which can be elicited by intramuscular injections of etiocholanolone. This was evaluated in more than 50 patients with various connective tissue diseases. The response was depressed in Felty's syndrome, in chronic active hepatitis, and in about half the patients with systemic lupus erythematosus. Two patients were restored to normal responsiveness by treatment. Auto-infusion of stored pretreatment plasma did not produce a fall in the baseline granulocyte count but resulted in an acute depression of the granulocyte response to etiocholanolone, a depression which gradually returned to normal over the ensuing 96 hours. (Drs. Talal, Decker, Plotz Wolff (I-LCI) and Kimball (I-LCI)).

Interest in and continuing admissions of patients with Sjogren's syndrome were employed to study the latent renal tubular acidosis which many of these hyperglobulinemic patients show. (Drs. Talal, Kaltreider, Zisman (H-CE), Schur (R.B.Brigham Hospital), Asofsky (I-LGAR) and Lightbody (D-OMS)).

II. Clinical Therapeutics

Therapeutic studies include work with Raynaud's phenomenon, chronic synovitis in rheumatoid arthritis and the nephritis of systemic lupus erythematosus. A variety of radiological and physiological parameters pertinent to the arterial circulation of the extremities are studied before and after the intra-arterial injection of reserpine in patients with painful finger tip ulcerations due to Raynaud's phenomenon. One patient has reversed and healed chronic indolent ulcers after a single injection. (Drs. Willerson, Decker, Thompson (A-MD), Herdt (CC-DR) and Hookman (D. C. General Hospital)). Prophylactic synovectomy of the knee continues under study. The short term results have been excellent. (Drs. Decker and Peterson (G. W. University Hospital)).

The work has resulted in the availability of a number of synovial specimens which have been assayed for collagenase activity after several days in tissue culture. Collagenase has been detected only in rheumatoid membranes and not in specimens from traumatic or degenerative joint disease surgery. Ranking of the rheumatoid arthritis patients by clinical disease activity and by severity grading of the synovial histology shows that higher values of collagenase activity are associated with more active and more severe rheumatoid arthritis. (Drs. Decker, Barth, Sokoloff (A-LEP), Hearon (A-MR) and Lazarus (D-LHP)).

The possibility that the immunosuppressive potential of chloramphenicol may benefit patients with systemic lupus erythematosus nephritis is under study. Progress is slow because we are anxious to use this potentially hazardous drug only where we are certain all other avenues have failed. (Drs. Talal, Plotz, Barth and Decker).

III. Animal Models of Disease

Work with possible animal models of amyloidosis, systemic lupus erythematosus, and rheumatoid arthritis is in various stages. Mouse amyloidosis can be induced by repeated casein injections. Using this system it has been possible to show changes in spleen ribosome function and reduced protein synthesis during the "inductive" phase. (Drs. Talal, Barth, Willerson and Asofsky (I-LGAR)). Amyloidosis has been transferred by the transfer of cells from a casein-treated donor to a healthy recipient and efforts to achieve such a transfer with subcellular fractions appear hopeful. (Drs. Barth, Willerson, Talal and Asofsky (I-LGAR)). The recent availability here of NZB and NZB/NZW F_1 mice has resulted in a series of new studies. It has been shown that these species, with their proclivity for autoimmunity and lupus-like pathological changes, are quite unable to develop tolerance to large doses of human or bovine gamma globulin in sharp contrast to other strains, lacking manifestations of autoimmunity. (Drs. Talal, Staples, Leventhal (C-MB), Sheagren (I-LCI) and Schur (R. B. Brigham Hospital)).

Contracts have been let for the maintenance of 30 young hogs. They are to serve as control and test animals for the intraperitoneal administration of Mycoplasma hyorrhinis, an organism which is expected to produce a non-purulent arthritis in some but not all animals. The developing immunity to the infecting agent will be serially tested looking for differences which can be correlated with the clinical response. (Drs. Soghor, Decker, Barden (I-LVD) and Hopps (DBS-LVI)).

IV. Nature of Antibodies

The synthesis of protein by the bound and free ribosomes of mouse spleen has been related to the structural and functional properties of the two types of particles. Bound ribosomes are more sensitive to conditions of preparation and incubation than are the free. Mouse spleen production of several classes of immunoglobulins has been shown the Jerne plaque technique. (Drs. Talal, Plotz and Kaltreider).

An area of interest has developed with the important discovery of antibody activity in a mouse myeloma protein. The first so identified possessed anti-dinitrophenyl group activity. The fortuitous combination of a genetically inbred host animal producing large amounts of homogeneous antibody to a chemically well-defined antigen provides a unique and powerful tool for the study of antibody structure-function relationships and antibody synthesis. (Drs. Metzger and Potter (C-LBGY)).

Structural and functional studies of a Waldenstrom macroglobulin with anti-gamma globulin activity have continued. In tests against the gamma globulins of several major families of primates, the antibody activity was strongest against those phylogenetically closest to man. The antibody could be totally precipitated by even the weakest antigen. The small scatter observed in binding-constant measurements using antibody active fragments of the macroglobulin suggested a high degree of functional homogeneity. Primary sequence studies are underway. Initially the N-terminal sequence of both the heavy and light chains will be compared to those of non-antibody macroglobulins. (Drs. Metzger, Stone and Kaplan).

In the course of the above work, a computational method for quantitating the differences between two proteins in reference to their amino acid composition has been developed. The difference index was found to correlate with the degree of relatedness as defined on phylogenetic or other bases. (Drs. Metzger, Shapiro, Mosimann and Vinton (last three doctors with CR-LAS)). In addition a new method, equilibrium molecular sieving, has been developed and applied to the interaction between a protein antigen and antibody; it has proven to be swift, convenient and reproducible. The method depends upon the differential absorption or penetration of differently labeled larger and smaller molecules into cross-linked gels. (Drs. Metzger and Stone).

V. Intermediary Metabolism

Studies of hypoxanthine-guanine phosphoribosyltransferase (H-GPRT) deficiency states have been extended to 20 patients with virtually complete deficiency and 16 with a partial deficiency. The former show the typical symptoms of choreoathetosis, spasticity, mental retardation, and compulsive self-mutilation. The latter show articular gout, urinary calculi, and, in some mild degrees of neurological dysfunction.

Fibroblasts grown in tissue culture from the mother of a boy with the complete deficiency were constituted of two populations, one with and the other without the enzyme. This is the first demonstration of the heterozygous state and provides evidence of the location of the pertinent gene on the X-chromosome.

The enzyme, H-GPRT, has been found in highest concentration in the brain, particularly the basal ganglia. Lack of the enzyme was found to be accompanied by a 4-to 5-fold excess of cerebrospinal fluid oxypurines above the normal level. (Drs. Seegmiller, Fujimoto, Greene, Friedman, Henderson (Univ. of Alberta), Caldwell (Univ. of Alberta) and Subak-Sharpe (Inst. of Virology, Scotlant)).

The adult and childhood forms of cystinosis were characterized by their high intracellular cystine content, higher in the juvenile form. Much smaller but quite definite increases in leukocyte and fibroblast cystine concentrations were found in the cells of the clinically normal parents of cystinotic children, thus providing the first biochemical definition of the heterozygous state. Penicillamine and low sulfur amino acid diets reduced plasma cystine concentration with neither clinical benefit nor reduction of the intracellular content. (Drs. Seegmiller, Friedman, Schneider (NINDB) and Wong (NINDB)).

VI. Biochemical Pharmacology

Enrichment culture techniques have permitted the harvesting of enzymes with remarkable specificity for various drugs, drug metabolites or drug analogues. The method has contributed to understanding the metabolic pathways of methotrexate. In addition it has produced a series of carboxypeptidases which are specific for the terminal amino acid which is removed. (Drs. Goldman and Levy (NCI)).

A series of studies of salicylate metabolism with reference to clinical efficacy have been begun. Further studies of the carbon-halogen bond in biology have been performed using enzymes developed through enrichment culture. An enzyme capable of breaking the carbon-fluorine bond showed dehalogenation reaction rates of F >Cl >I, the opposite of predicted activity based on bond energies. As more is learned of the mechanism of fission of the carbon-halogen bond, analogous mammalian systems will be studied. (Drs. Goldman and Milne (NHI)).

Serial No. NIAMD-ARB-1c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of Infection in Rheumatic Disease

Previous Serial Number: Same

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. David Soghor, Dr. Jerri Barden (I-LVD) and Dr. Hope Hopps (DBS-LVI).

Cooperating Units: National Institute of Allergy and Infectious Diseases, Laboratory of Viral Disease and Division of Biologics Standards, Laboratory of Viral Immunology.

Man Years:

Total:	1-3/4
Professional:	1
Other	3/4

Project Description:

Objectives:

The overall object is to discover the cause of rheumatoid arthritis. To this end we are pursuing two types of microbiological agents which are believed to be associated with mammalian synovitis - mycoplasma and Bedsonia. In addition we are making efforts to measure and thus understand the role of host responsiveness in infection.

Methods Employed:

Within the last two months a contract has been let for the maintenance of a group of 30 young specific-pathogen-free swine. In a planned pilot project these animals are to be innoculated with mycoplasma hyorrhinis and their subsequent course closely followed. We plan synovial fluid studies, reisolation attempts, and histopathological examination of both the control and experimental series. Procine white cell responsiveness and skin reactivity to the infecting agents will be studied. Efforts will be made to distinguish the reactivity of the two groups expected to appear in the infected group - those with and those without arthritis.

Patients with fresh Reiter's syndrome have been admitted to the Clinical Center or seen in other local hospitals. Serum and synovial fluid specimens have been obtained for Bedsonia complement fixation tests and attempts at Bedsonia isolation in chicken yolk sac.

White blood cell (both macrophage and lymphocyte) reactivity have been assayed in essentially two systems. Early in the report period this was being done by lymphocyte transformation and thymidine uptake. More recently we have been examining the leukocyte migration from capillary tubes out into media containing or not containing specific antigens under test such as tuberculin.

Major Findings:

Preliminary efforts to identify herds of swine free of mycoplasma hyorrhinis proved more difficult than anticipated; the organism seems to be a normal inhabitant of the nasopharynx. The experimental groups are to be delivered by section and raised by hand so that their first contact with the agent will be under controlled circumstances.

Specimens from six patients with Reiter's syndrome have been studied. Two of these were fresh and had received no antibiotic. We have failed to isolate Bedsonia entirely - results which are consistent with those of several other laboratories.

The white cell migration system has proven to be remarkably reproducible and changes on the order of 30 to 40% inhibition of migration have resulted when specific antigens are included in the media. The relationship of this response to tuberculin type delayed hypersensitivity has not as yet been sharply delineated but the system seems to be so sensitive that it is modified by recent skin testing of the white cell donor.

Significance:

Among many other hints, the current interpretations of rheumatoid factor emphasize its probable relationship to undetected infection. If we assume that rheumatoid arthritis is related to an exogenous agent, epidemiological information implies that the agent must be very widespread and suggests that every human has been thoroughly exposed. On these assumptions, the importance of host reactions to agents alternately capable and incapable of inducing chronic arthritis is self evident.

Proposed Course:

The leukocyte and Bedsonia work will continue as at present.

The swine arthritis will require a major effort through the next few months.

Honors: None

Publications: None

Serial No. NIAMD-ARB-2c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Prophylactic Synovectomy in Rheumatoid Arthritis

Previous Serial Number: Same

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Leonard T. Peterson, Orthopedic Consultant, George Washington University School of Medicine

Cooperating Units: George Washington University School of Medicine, Washington, D.C.

Man Years:

Total:	3/4
Professional:	1/4
Other:	1/2

Project Description:

Objectives:

To discover whether or not prophylactic synovectomy, as defined below, will alter the functional or structural prognosis in rheumatoid arthritis.

Methods Employed:

Patients with rheumatoid arthritis who have had at least six months of active inflammation in both of a pair of symmetrical joints, neither of which show radiological changes other than osteoporosis, have been admitted to the study. The disease must be sufficiently symmetrical to have all parties -- the investigator, the surgeon, and the patient -- agree to have either one of the pair operated. The side to be done is selected by lot.

The patients are then seen at intervals of six months, one year, and two years. On these visits they undergo standard evaluation for function and radiological examination.

Major Findings:

Four patients have undergone unilateral synovectomy in this report period raising the total in the study to 11 cases. The early results continue excellent and no complications of surgery have been observed. Regular followup evaluations continue.

Significance to Bio-Medical Research and the Program of the Institute:

There has been essentially no controlled work on this important therapeutic modality which is being widely urged on the basis of inadequate data. There is reason to believe that, almost alone among treatment methods, it will prevent joint damage. This needs to be established.

Proposed Course:

The study will continue.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-3c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in Raynaud's Phenomenon

Previous Serial Number: None

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. James T. Willerson, Dr. Ronald Thompson (A-MD), Dr. Jean Herdt (CC-DR), and Dr. Perry Hookman (D. C. General Hospital).

Cooperating Units: National Institute of Arthritis and Metabolic Diseases, Metabolic Chamber Team; Clinical Center, Diagnostic Radiology Department; D. C. General Hospital.

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

To develop methods for the control of Raynaud's phenomenon and for the prevention of the characteristic, disabling fingertip ulcers. The approach also permits us to entertain the hope of understanding the pathophysiology of the process.

Methods Employed:

Patients with either the primary or secondary form of the disease are being admitted to the clinical service. After general evaluation, they undergo a series of tests including brachial arteriography, esophageal pressure recording, venous plethysmography and finger tip heat transfer studies. Some of these are performed both in the normal resting state and while the patient is actually suffering a spontaneous or cold induced attack.

After establishing baseline performance levels the patients are given intra-arterial reserpine as a single dose or they are begun on systemic reserpine. The observations are then repeated.

Major Findings:

Seven patients have completed various parts of this protocol and continue under study. We have not been impressed by the relationship of Raynaud's phenomenon and esophageal aperistalsis as reported by others.

Two patients with intractable finger tip ulcers have received intra-arterial reserpine. One of these began an immediate improvement, cleared the lesion completely and is ulcer-free three months later. In the other individual there was some improvement but not healing.

Significance to Bio-Medical Research and the Program of the Institute:

There is no available explanation for the fact that Raynaud's phenomenon so frequently precedes or accompanies the connective tissue diseases. The vasomotor instability shown by these patients may be related to vasoactive amines or peptides which are beginning to be recognized as playing a role in inflammation. We believe that understanding of Raynaud's would put us closer to understanding connective tissue disease. In the interim, the symptoms of the uncomplicated process can be incapacitating and their prospective relief is worthy of study in its own right.

Proposed Course:

The studies will continue as outlined.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-4c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunological Reactivity in the Elderly

Previous Serial Number: Same

Principal Investigator: Dr. John L. Decker

Other Investigators: Drs. Robert F. Willkens (University of Washington) and Donald S. Waldorf (formerly NCI) (New York University)

Cooperating Units: Division of Arthritis, Department of Medicine University of Washington, Seattle, Washington and National Cancer Institute, Dermatology Branch.

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

The objectives, methods, findings and significance of this work have been previously reported and the project terminated.

Honors and Awards: None

Publications:

Waldorf, D.S., Willkens, R.F., and Decker, J.L.: Impaired delayed hypersensitivity in an aging population; association with antinuclear reactivity and rheumatoid factor. J.A.M.A. 203: 831-834, (Mar. 4) 1968.

Serial No. NIAMD-ARB-5c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Synovial Membrane Collagenase

Previous Serial Number: None

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Werner Barth, Dr. Leon Sokoloff (A-LEP), Dr. John Hearon (A-MR), and Dr. Gerald Lazarus (D-LHP)

Cooperating Units: National Institute of Arthritis and Metabolic Diseases, Laboratory of Experimental Pathology; National Institute of Arthritis and Metabolic Diseases, Mathematical Research Branch; National Institute of Dental Research, Laboratory of Histology and Pathology.

Man Years:

Total:	1-1/2
Professional:	3/4
Other:	3/4

Project Description:

Objectives:

Dr. Lazarus, using synovial material obtained through this branch and several other Arthritis units, has been able to show the development in synovial tissue culture of a highly specific collagenase active against native collagen at physiologic pH and temperature ranges. Specimens from rheumatoid arthritic patients showed activity but specimens from patients with traumatic conditions and degenerative joint disease did not.

The purpose of this work was to correlate the amount of collagenase produced with both systemic and local disease activity of the synovial donor.

Methods Employed:

The patients were retrospectively ranked in order of their disease activity without knowledge of the collagenase produced. Clinical activity determinations were based upon number of joints painful on motion, medications in use, duration of morning stiffness, and erythrocyte sedimentation rate at the time of surgery.

Local disease activity was reduced to numerical values by grading six histological features of active rheumatoid synovitis on a 0 to 3+ scale. The grades of each specimen were summed to produce disease activity index.

Major Findings:

The statistical analyses are nearing completion. In outline it is clear that more collagenase is produced by specimens showing high local disease activity and/or more systemic disease activity. The former appears most closely correlated. The correlation is not due exclusively to cellularity as measured by total deoxyribonucleic acid content of the tissue. The data show a strongly positive correlation between degree of tissue vascularity and collagenase production.

Significance to Bio-Medical Research and the Program of the Institute:

The local and crippling effects of rheumatoid arthritis are due in large part to the loss of structural collagen from bone, cartilage, ligaments, and tendons. The removal of collagen must be initiated, at least, by collagenase. Thus we regard knowledge of the control of the enzyme as of great importance.

There are certain clinical types of rheumatoid arthritis in which long persistent, active soft tissue joint inflammation does not result in structural loss. It will be of importance to compare collagenase production, release, and possible inhibition in such tissues as contrasted to those features in the usual rheumatoid synovia which has already extensively destroyed bone and cartilage.

Proposed Course:

This work will not be further pursued here because Dr. Lazarus is leaving the U.S.P.H.S.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-6c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Septic Arthritis

Previous Serial Number: None

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Werner Barth and
Dr. James T. Willerson

Cooperating Units: None

Man Years:

Total:	1-1/2
Professional:	3/4
Other:	3/4

Project Description:

Objectives:

To define the prodromal features and characteristics of septic arthritis with a view to establishing earlier diagnosis and treatment.

Methods Employed:

The work is based on the Clinical Center records of patients with demonstrated septic arthritis. These have been identified through the Medical Records Department, the Department of Pathology and through branch records and indices.

The records have been reviewed in detail.

Major Findings:

The effort to locate other cases was stimulated by an unusual case admitted to our service. The patient, on adrenal corticosteroid therapy for her rheumatoid arthritis, suffered a cat scratch of the calf. Cellulitis and subsequent local extension of the process to her chronically diseased knee was observed despite multiple courses of antibiotics. The infecting organism, Pasteurella multocida, was repeatedly isolated both from the patient and from the cat. A detailed report of the case and a remarkably similar additional case has been prepared.

Thirteen other cases of septic arthritis have been identified and their records are under study.

Significance to Bio-Medical Research and the Program of the Institute:

The failure to recognize this disease is tragic because of its reversibility, given early diagnosis. Accordingly we believe it important to study and publicize the earliest features as well as the clinical setting in which it develops.

Proposed Course:

The Clinical Center series will be the subject of a report. No further work is currently contemplated.

Honors and Awards: None

Publications:

1. Barth, Werner F., Healey, L. A. and Decker, John L.: Septic Arthritis Due to Pasteurella Multocida Complicating Rheumatoid Arthritis. Arth. and Rheum. (In press).

Serial No. NIAMD-ARB-7c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Antibody and Protein Synthesis in the Spleen

Previous Serial Number: Same

Principal Investigator: Dr. Norman Talal

Other Investigators: Dr. Paul Plotz and Dr. Henry Kaltreider

Cooperating Units: None

Man Years:

Total:	1-1/4
Professional:	1
Other:	1/4

Project Description:

Objectives:

Study of the biochemical events underlying the synthesis of antibody, with emphasis on the functional properties of spleen ribosomes.

Methods Employed:

1. Standard techniques of cell fractionation, sucrose density gradient analysis, isotope incorporation by spleen fragments and cell-free system, analysis of RNA and base ratios.

2. Enzymatic digestion and analysis by chromatography and electrophoresis of isotopically labeled products of cell-free synthesis.

3. Detection of spleen cells producing sheep hemolysins by direct and indirect Jerne plaque technique.

Major Findings:

A. The functional and structural properties of deoxycholate - liberated bound and free ribosomes from rat spleen have been compared. The efficiency with which natural and particularly synthetic messenger RNA is translated depends greatly upon the conditions of preparation and incubation. Bound ribosomes are more sensitive to these conditions than are the free. Both ribosomes have similar sedimentation coefficients, contents of RNA and nucleotide bases, and patterns of polyribosomes, structural and rapidly labeled RNA. They are immunologically identical but have different banding patterns in disc gel electrophoresis.

B. During the development of casein-induced splenic amyloidosis in C_3H mice, protein synthesis and ribosome function undergo a biphasic alteration. There is a maximum impairment just before amyloid is deposited followed by a return towards normal. The translation of synthetic messenger by maximally impaired ribosomes in only 25% of controls. This is only in part due to a reduction in messenger binding, and suggests the presence of a factor regulating translation. Control ribosomes from animals injected with gelatin, Freund's adjuvant and phytohemagglutinin show no impairment. These functionally depressed ribosomes may initiate the synthesis of amyloid protein.

C. Cells producing all classes of immunoglobulin antibodies except IgA have been identified in mouse spleen by the indirect Jerne plaque assay. Two classes of mouse IgM antibodies differing in their ability to fix complement have been found in serum.

Significance to Bio-Medical Research and the Program of the Institute:

The problem of spleen protein synthesis is intriguing for many reasons. From the standpoint of molecular biology, an understanding of antibody synthesis may contribute some new insights into the general mechanism of protein formation. From the clinical standpoint, these studies may shed some light on the pathogenesis of amyloidosis.

Proposed Course:

A. Continued investigation of the ribosomal alteration in casein-induced amyloidosis to elucidate its biochemical basis.

B. Study of bound and free ribosomes in immunologically stimulated animals to look for factors regulating translation.

C. Attempt by peptide analysis to demonstrate synthesis of immunoglobulin.

Honors and Awards: None

Publications:

1. Plotz, P. H. and Talal, N. Fractionation of splenic antibody-forming cells on glass bead columns. J. Immunol. 99:1236-1242, 1967.

2. Plotz, P. H., Talal, N. and Asofsky, R. Assignment of direct and facilitated hemolytic plaques in mice to specific immunoglobulin classes. J. Immunol. (In press).

3. Plotz, P. H., Colten, H. and Talal, N. Mouse macroglobulin antibody to sheep erythrocytes: A non-complement fixing type. J. Immunol. (In press).

Serial No. NIAMD-ARB-8c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Structural Analysis of a Human Macroglobulin

Previous Serial Number: Same

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Marvin J. Stone and Dr. Allan Kaplan

Cooperating Units: None

Man Years:

Total:	2-1/4
Professional:	1-3/4
Other:	1/2

Project Description:

Objectives:

We are continuing our study of human macroglobulins (γM) and the relationship of their structure to that of human (γG) immunoglobulins. During the past year we have begun to do some selective sequence analysis on one of the macroglobulins. In addition a major interest has evolved in an analysis of a Waldenstrom with antibody activity.

Methods Employed:

In addition to the techniques of immunochemistry and protein chemistry previously employed in the laboratory we are beginning to acquire the apparatus and techniques to do primary sequence analysis.

Major Findings:

I. The valency of macroglobulin antibodies.

Although previous studies from our own laboratory (Miller and Metzger, J. Biol. Chem. 240:3325, 1965; ibid 240:4740, 1965; ibid 241:1732, 1966; ibid 241:1741, 1966) now confirmed by other laboratories has indicated the similarity of the basic structure of γM immunoglobulins to that of γG immunoglobulins, considerable question has remained about the valency of the γM immunoglobulins. This is an important problem since if the subunit were univalent (as some have claimed) it might imply asymmetry of the molecule which in turn would raise interesting biosynthetic problems. The availability of a Waldenstrom macroglobulin possessing bonafide antibody activity (Metzger, Proc. National Acad. Sci. 57:1490, 1967) has permitted us to reexamine this problem. Experiments were conducted in which the Fabμ fragments from the antibody were prepared as previously described and examined in the analytical ultracentrifuge in the presence of γG immunoglobulin (the antigen). By examining the degree to which the Fabμ peak disappeared we could calculate the percentage of these fragments which were active. It was found that over 90% of the Fabμ fragments were capable of specifically reacting with the antigen, thus, proving that all of the Fabμ fragments obtainable from a γM macroglobulin have a similar combining site. We believe this to be the first conclusive evidence of the deca-valence of γM immunoglobulins. It was interesting in this respect to reexamine the valency of the subunit as opposed to the Fabμ fragments. In these experiments tryptic Fc fragments were prepared from a human γG myeloma and these were run in the centrifuge in the presence of the subunit of the macroglobulin (γM_S). At saturating levels of the Fc fragment only one mole of the Fc fragment was bound per mole of subunit. These experiments seemed to indicate that the failure of others, as well as ourselves, to find the expected valence of the subunit may be due to steric inhibition of the combining sites as they are present in subunit rather than due to any asymmetry of that subunit.

II. The functional properties of a human Waldenstrom macroglobulin antibody.

The discovery of paraproteins possessing antibody activity has permitted one, for the first time, to examine the properties of potentially homogeneous antibodies. We have examined several aspects of this problem with respect to the Waldenstrom macroglobulin antibody which we have been working with.

a. The specificity of the Waldenstrom macroglobulin antibody.

We have examined the specificity of the antibody we are working with by studying its reaction with γG immunoglobulins obtained from the major families of primates. The results indicate the following: 1) the antibody appears to be highly specific, reacting best with those immunoglobulins most closely related phylogenetically to human immunoglobulins and to a lesser extent or not at all with immunoglobulins from species more distantly related. 2) Despite the above observation it is clear that the antibody does not possess the remarkable discriminatory powers of a mixture of antihuman γG antibodies such as found in a rabbit antiserum. 3) Even with the most weakly cross-reacting primate immunoglobulin the macroglobulin antibody can be totally precipitated.

These results suggest that the antibody may be reacting with a single determinant on the γG immunoglobulin.

b. The functional homogeneity of the Waldenstrom antibody.

The functional homogeneity of the macroglobulin antibody was examined in experiments in which the binding constant of the antibody for the antigen could be directly assessed. These studies were performed by studying the interaction of Fabμ fragments from the antibody with the γG antigen in the analytical ultracentrifuge and, in addition, by a new method which we have called equilibrium molecular sieving (see separate Project Report). In brief, these experiments indicated that the binding constant of the macroglobulin is approximately 2×10^4. We have been able to study the binding constant over a range of binding in which 10-90% of the sites were reacted. Despite a small amount of scatter to the data it is clear that the binding constant appears to be the same for all the Fab fragments, thus attesting to the functional homogeneity of this antibody.

c. Comparison of the activity of the macroglobulin in a homogeneous system as opposed to a multi-phase system.

In a standard precipitating system composed of the γM macroglobulin-polymer and human γG, it was found that the reaction was extremely sensitive to solvent conditions and temperature. Excellent precipitation is obtained at low temperatures, low ionic strength and pH's between 5.5 and 6.5, raising the temperature, the ionic strength and either lowering the pH or raising the pH above these limits, drastically decreases the precipitability of the macroglobulin. Since these features of the precipitant reaction are quite unusual when compared to ordinary antibodies it was of some interest to ascertain whether this was due to the primary antigen-antibody reaction or due to some secondary phenomena. In the equilibrium studies mentioned above, it was found by direct measurement that the association constant was quite insensitive to solvent condition. Thus the primary antigen-antibody reaction was very similar to the reaction of ordinary antibodies. It is therefore concluded that the unusual properties of the precipitating system are related to secondary phenomena and reflect both the homogeneity of the antibody and the univalence of the antigen. That is, it seems clear that the precipitation reaction is simply caused by the insolubility of the complexes of the γM antibody with the γG rather than due to complex matrix formation as is the case with heterogeneous antibodies reacting with multivalent antigens.

d. Primary sequence studies.

Significance to Bio-Medical Research and the Program of the Institute:

Paraprotein antibodies has been discussed in our previous Project Report. As is clear from the above report the paraprotein antibodies allow one the unique opportunity for studying the reactivity of isolated antibodies and therefore of getting increased insight into structural functional relationships of immunoglobulins.

The Waldenstrom macroglobulin antibody gives one the first opportunity to study the primary sequence of an antibody of known function. A preliminary examination to see whether the N-terminal sequences of the light and heavy chains from this macroglobulin are in any clearly defined way dissimilar from the same sequences obtained from macroglobulins not possessing this activity is under way. It will be possible to look at approximately the first 20 amino acids of both the kappa and the μ chain and to compare these sequences with the corresponding chains of other macroglobulins that we have in our possession as well as with light and heavy chain sequences from immunoglobulins which are avialable from the literature. It is too early at this stage to evaluate our findings.

Honors and Awards: None

Publications:

1. Stone, M. J., Metzger, H.: The Valency of a Waldenstrom Macroglobulin Antibody and Further Thoughts on the Significance of Paraprotein Antibodies. Cold Spring Harbor Symposium of Quantitative Biology, 32:83, 1967.

Serial No. NIAMD-ARB-9c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Leukopenia in Connective Tissue Disease

Previous Serial Number: Same

Principal Investigator: Dr. Norman Talal

Other Investigators: Drs. John L. Decker, Sheldon Wolff (I-LCI)
Harry Kimball (I-LCI), Paul Plotz

Cooperating Units: National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation.

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

Define the pathogenesis of the leukopenia that frequently accompanies rheumatoid arthritis, systemic lupus erythematosus and Sjogren's syndrome.

Methods Employed:

1. Measurement of "granulocyte response" (polymorphonuclear increase after administration of 0.1 mg/kg of etiocholanolone); routine bone marrow aspiration.

2. Auto-infusion of stored pre-treatment plasma in selected patients whose granulocyte response was restored to normal by therapy.

Major Findings:

Over 50 patients have been studied with etiocholanolone. The granulocyte response is generally normal in uncomplicated rheumatoid arthritis and Sjogren's syndrome. It is depressed in all patients studied with either rheumatoid arthritis and splenomegaly or with chronic active hepatitis, and in approximately half of all patients with systemic lupus erythematosus. It does not correlate with the baseline granulocyte count.

Two patients (one with rheumatoid arthritis and splenomegaly and one with chronic active hepatitis) were restored to a normal baseline granulocyte count and granulocyte response after splenectomy and chemotherapy respectively. Auto-infusion of stored pre-treatment plasma resulted in an acute depression of the granulocyte response which then returned to normal over the ensuing 96 hours. There was no significant fall in the baseline granulocyte count.

Significance to Bio-Medical Research and the Program of the Institute:

This study has two purposes: (1) to arrive at a concept for the pathogenesis of the leukopenia that occurs in the connective tissue diseases and (2) to possibly serve as an additional diagnostic test to distinguish between rheumatoid arthritis and systemic lupus erythematosus.

Proposed Course:

Continued evaluation of marrow reserve in these diseases, with particular emphasis on the presumed serum factor responsible for a depressed granulocyte response.

Honors and Awards: None

Publication: None

Serial No. NIAMD-ARB-10c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Projgect Report
July 1, 1967 through June 30, 1968

Project Title: Assessment of Compositional Relatedness Between Proteins

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Marvin Shapiro, Dr. James E. Mosimann, and Dr. Jay E. Vinton

Cooperating Units: Computer Systems Laboratory of the Division of Computer Research and Technology.

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

It is sometimes desirable to study the possible relationship of two proteins at a time when sequence data is unavailable. A possible useful criteria is amino-acid composition. In the present work a simple method was developed for assessing such compositional relatedness.

Methods Employed:

The mole fraction of each amino-acid for the proteins to be compared is calculated. The absolute difference between the mole fractions for each amino-acid is determined and the sum of the absolute differences is multiplied by fifty. In this way two proteins having identical amino-acid compositions will have a difference index of zero whereas two proteins having no amino-acid in common would have a theoretical difference index of 100. 630 comparisons between putatively unrelated proteins were made using a computer program. A distribution curve of these differences indexes was then plotted. When the difference index for proteins which are known to be related was compared with this distribution curve, it was clear that the difference index provided a meaningful indicator of such relatedness.

Significance to Bio-medical Research and the Program of the Institute:

It is clear that the difference index provides another useful criteria for studying the relatedness of proteins for which complete amino-acid sequence data is not available. While in of itself this index cannot prove or disprove such relatedness it can provide a useful criteria on which to base further investigation. This computational method was recently applied by Steers and Marcesi for purposes of comparing a red-cell membrane protein, muscle actin and a cilary protein. Their preliminary data which suggested that there might be a relationship between these three proteins appears to be strongly substantiated by the low difference index and provides a valid example of its use.

Proposed Course:

A manuscript has been prepared and is ready for submission. The project is otherwise complete.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-11c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Treatment of Lupus Nephritis with Chloramphenicol

Previous Serial Number: Same

Principal Investigator: Dr. Norman Talal

Other Investigators: Drs. John L. Decker, Paul H. Plotz and Werner Barth

Cooperating Units: None

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

The best available evidence suggests that lupus renal disease is due to precipitation of specific immune complexes in the glomeruli. The current treatment of this condition leaves much to be desired. The ability of chloramphenicol to suppress the immune response has been shown in several experimental systems. This pilot study in a limited number of patients was undertaken to evaluate the efficacy of chloramphenicol as an immunosuppressive drug in the treatment of lupus renal disease.

Methods Employed:

Routine serum and urine studies; measurement of biologically active and total chloramphenicol in serum (performed by Parke, Davis and Co.); renal biopsy if deemed advisable.

Major Findings:

Only one patient has been treated. The drug (2 gm daily) was discontinued near the end of the one-month course because of hematologic toxicity. Although there was no objective evidence of a therapeutic response as defined in our protocol, this patient has done better than predicted in the followup period. This apparent remission of severe lupus nephritis and nephrotic syndrome warrants further evaluation.

Significance to Bio-Medical Research and the Program of the Institute:

This study is designed to evaluate a commonly used antibiotic, chloramphenicol, as a possible agent in the treatment of the often fatal nephritis that accompanies systemic lupus erythematosus.

Proposed Course:

Continued evaluation in a total of ten patients.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-12c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on a Mouse Myeloma Protein Having Antibody Activity

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Michael Potter

Cooperating Units: Laboratory of Biology, National Cancer Institute

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

It is now clear that paraprotein antibodies do exist. The initial two proteins which have been described which possess such activity were of human origin. The discovery that such antibody activity can also be found among mouse myeloma proteins provides one the unique opportunity not only to study homogeneous antibodies but to study such antibodies arising in genetically inbred strain. It is clear that study of these materials provides a tremendous break-through both with respect to structural-functional relationships of antibodies and to the biosynthesis of antibodies.

Methods Employed:

Our initial studies have been directed to the study of the antibody combining site in such a myeloma protein possessing antibody activity directed towards the dinitrophenyl group. Studies previously conducted on rabbit anti-DNP antibodies (Metzger, et al., Biochemistry 2:979, 1963) are now being applied to the study of this mouse myeloma antibody.

Major Findings:

This project has been worked on for only three months but a few interesting results have already developed.

1. It is clear that the labelling of the combining site can be affected by a variety of reagents; the labelling being highly specific.

2. Unlike all previous studies the specific labelling appears to be exclusively on one of the two types of immunoglobulins chains, in this case the light chain.

3. Preliminary data suggest that each of the combining sites capable of binding hapten is also capable of being specifically labelled. This is in marked contradistinction to the case of the rabbit antibodies which were studied where approximately only a quarter of the combining sites were capable of being specifically labelled.

Significance to Biomedical Research and the Program of the Institute:

The discovery of the mouse myeloma proteins possessing antibody activity provide a significant advance in the materials available for studying the nature of antibody specificity as well as the synthesis and control of antibody production. The entire immunochemical literature is based on results with heterogeneous antibodies. It is clear that many of the fundamental results must now be reevaluated, using the homogeneous antibodies which are now available.

Proposed Course:

It is too early to decide which direction our own laboratory shall pursue in studying these molecules. One study which is immediately possible is the isolation of the labelled peptides from the combining site, sequencing of the peptides and ascertaining their position in the immunoglobulin chain. A number of different labelling reagents are being evaluated with the hope that by the use of different reagents different amino-acids contributing to the combining sites can be delineated.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-13c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of Macromolecular Interactions by Equilibrium Molecular Sieving

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Marvin Stone

Cooperating Units: None

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

In our studies with a Waldenstrom macroglobulin antibody it became necessary to study the interaction of two large proteins, one having a molecular weight of 50,000 and the other a molecular weight of 150,000. We wished to have a technique by which this interaction could be rapidly and conveniently assessed. In the absence of other good methods for studying such an interaction we decided to develop a technique which we have called equilibrium molecular sieving.

Methods Employed:

The technique employed can be summarized simply as follows. The cross-linked dextran or polyacrylamide gels commonly used for molecular sieving on columns have the unique property of permitting differential penetration of macromolecules approximately according to their size. These cross-linked gels can therefore be thought of as membranes of varying porosity. The gels have been used previously for studying macromolecular interactions but only by dynamic (column) procedures. The theory for such a dynamic system is complicated and involves assumptions which at times are difficult to justify. Most importantly for our purposes each determination requires a column run so that the method is time-consuming and inconvenient. Several studies have appeared in which a closed tube (static) system was used for studying macromolecule-small molecule interactions but the technique has not been previously applied to macromolecule macromolecule interactions. Moreover, the previous studies have been done under conditions where the association constant of the species being studied was extremely high and where volume measurements did not require extreme precision. In our own studies, because of the weak binding constant (2x104) as well as other constraints, we have had to develop a method by which precise volume measurements could be made. This was accomplished using a variety of differentially labelled markers. In practice, the method involves filling a small glass tube with approximately 2 ml of a Sephadex slurry, removing any supernatant fluid, adding approximately 0.2 ml of the starting solution and then mixing the tube by inversion for a short period of time. The gel is allowed to settle and an aliquot of the supernatant removed. By studying the concentration of the various reactants compared to the markers in both the starting solution and the supernatant of the tube the fractional extent of binding can be directly determined. Furthermore, since the amounts of each of the reactants added to the tube of Sephadex is known and their distribution volumes directly determinable, an association constant can be simply calculated.

Major Findings:

In brief, the major finding was that this method is ideally suited for studying certain types of macromolecular interactions. In our own case, we studied the binding of the Fabμ fragment (molecular weight 50,000) to human γG (molecular weight 150,000). Under the conditions used, equilibration was extremely rapid, taking less than 15 minutes. We were able to study the binding constant over a large range of saturation and to evaluate the effects of temperature and various solvent conditions. It was possible to determine the association constant for the system and the various thermodynamic parameters of the interaction. One of the reasons for developing this method was for the purpose of using it as an assay system in an attempt to isolate the antigenic determinant present on γG. That is, by breaking down the Fc fragment of the γG and obtaining small peptides, these could be studied as potential inhibitors of the interaction. The ease of setting up multiple points using the Sephadex system would make it an ideal method for this kind of assay.

Significance to biomedical research and the program of the institute:

We feel that the technique developed may be of wide usefulness for studying macromolecular interactions. The method is rapid and convenient and does not require any complicated apparatus.

Proposed Course:

This project has now been completed.

Honors and Awards: None

Publications:
A manuscript on this subject is ready for submission.

Serial No. NIAMD-ARB-14c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Human Primary Systemic Amyloidosis: Clinical, Immunochemical and Metabolic Studies

Previous Serial Number: Same

Principal Investigator: Dr. Werner F. Barth

Other Investigators: Drs. James T. Willerson, T. Waldmann (NCI), and Robert Zelis (NHI).

Cooperating Units: National Cancer Institute, Metabolic Branch; National Heart Institute, Cardiology Branch.

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

Much clinical and experimental work has suggested a relationship between immunoglobulins and primary systemic amyloidosis. One hypothesis has suggested that all patients with primary amyloidosis have Bence Jones proteinuria and that this is pathogenetically significant in its synthesis and deposition. Our objective was to study patients with primary amyloidosis and determine whether or not Bence Jones proteinuria was present. We also hope to perform immunoglobulin metabolism studies in order to better define their relationship to amyloidosis.

Methods Employed:

Concentrated urine and serum were examined by immunoelectrophoresis with specific anti-light chain antibodies to determine the presence or absence of Bence Jones proteinuria. Immunoglobulin preparations of IgG, IgA, and IgM were labeled with I^{131} or I^{125} and metabolic studies performed.

Major Findings:

Only 6 of 15 patients with primary amyloidosis studied were found to have

Bence Jones proteinuria. In only 9 of 15 patients was there either an anomolous serum and/or urinary immunoglobulin. Immunoglobulin metabolism in patients with serum paraproteins and Bence Jones proteinuria showed decreased synthesis of immunoglobulins. The patients with an anomolous serum and/or urinary immunoglobulin were clinically distinguishable from those without an immunoglobulin abnormality. Macroglossia, carpal tunnel syndrome and amyloid purpura were found only in the patients with an anomolous immunoglobulin.

Peripheral blood flow studies were performed in nine patients with and without extensive systemic vascular involvement. Results of the study suggest that the blood vessels lose their ability to dilate or respond to vassdilator stimuli when extensive vascular involvement is present.

Significance to Bio-Medical Research and the Program of the Institute:

The absence of Bence Jones proteinuria in a large number of patients with primary amyloidosis doesn't support the hypothesis that Bence Jones proteinuria is of pathogenetic significance in the disease. If there is a relationship, it does not appear to be a direct one. Our results also suggest a heterogeneity in patients with primary amyloidosis, not previously appreciated. The synthesis of immunoglobulins in patients with amyloidosis and a paraprotein is much less than that observed in cases of multiple myeloma and suggests that a different mechanism or control is present.

Proposed Course:

It is hoped that this study can be continued to determine whether the observations are valid in a larger series of patients. We would also like to determine whether macroglossia, carpal tunnel syndrome, and purpura are useful clinical indicator of the presence of an anomolous protein. Continued studies of immunoglobulin synthesis, immunofluorescent studies of the bone marrow, and *in vitro* studies of immunoglobulin synthesis by marrow plasma cells are indicated.

Honors and Awards: None

Publications:

1. Barth, W. F.: Amyloidosis Review of Cardiac and Renal Manifestations. Med. Ann. D.C. 36, 228-231, 1967.

Serial No. NIAMD-ARB-15c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Experimental Mouse Amyloidosis

Previous Serial Number: Same

Principal Investigator: Dr. Werner F. Barth

Other Investigators: Drs. James Willerson, Norman Talal, and Richard Asofsky, (NIAMD-LGAR)

Cooperating Units: National Institute of Arthritis and Metabolic Diseases, Laboratory of Germ Free Animal Research.

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

Repeated injections of casein into the mouse has long been used as a model of inducing amyloidosis. The mechanism is completely unknown and the results in the literature contradictory and difficult to interpret. Three years ago, we set out to study various aspects of this model system in order to learn more about the mechanism whereby casein induces amyloidosis and therefore, something of the pathogenesis of amyloidosis.

Methods Employed:

Mice were injected daily with casein and sacrificed at appropriate points in time depending on the nature of the experiments. Ribosome function in casein treated and control mice was studied *in vitro* in isolated ribosomes. Immunoglobulin levels were determined using specific mouse antisera and immunodiffusion plates. Experiments of amyloid transfer were performed using spleen cells isolated from casein treated mice at appropriate points in time.

Major Findings:

1. Repeated injections of casein into mice reproducibly induce an alteration in ribosome function during the *inductive* stages of amyloid formation. This alteration is characterized by a decreased synthesis of protein as determined by

labeled leucine incorporation and a marked depression of ribosome function when stimulated with synthetic messenger (poly U). Studies using other forms of stimulation (gelatin, Freunds adjuvant) indicate some specificity to the casein response.

2. We found that the response to casein administration was different in each of six mouse strains examined. In two strains amyloidosis was quickly and easily induced whereas in one strain amyloidosis was very difficult to induce.

3. The immunoglobulin response was studied during amyloid induction and development. The major immunoglobulin increase was in only one of the five mouse immunoglobulin classes, and the response qualitatively similar to that seen after Freunds adjuvant administration. The repeated injection of this mouse immunoglobulin class to mice to achieve levels comparable to those after casein administration did not induce amyloidosis.

4. The effect of cytostatic drugs (cytoxan, nitrogen mustard and x-irradiation) on amyloid formation was studied. Preliminary results suggest that amyloidosis is accentuated only if amyloid formation is already in process.

5. Amyloidosis has been successfully transferred from a donor to an untreated recipient. The conditions for this have now been defined.

Significance to Bio-Medical Research and the Program of the Insititute:

The ribosome studies for the first time demonstrate an abnormal biochemical phenomenon during the early and inductive stages of amyloid formation. It is possible that this is due to a cofactor deficiency or enzymatic defect and may specifically relate to the subsequent deposition of amyloid.

The strain experiment suggests that genetic factors are involved in the susceptibility and ultimate development of amyloid.

Despite the marked hyperglobulinemia induced by casein, the absence of amyloidosis following administration of gamma globulin in comparable doses is the first clear cut demonstration that amyloidosis and hyperglobulinemia are not directly related.

The availability of a model to study amyloid transfer now offers an opportunity to study the particular cell and subcellular factors involved.

These various studies have permitted us to construct a working hypothesis of amyloid induction which is amenable to further study.

Proposed Course:

We hope to continue these experiments to better understand the mouse model and learn more of the pathogenesis of amyloidosis. We hope to demonstrate specificity of the ribosomal abnormality using another mode of amyloid induction. The experiments on amyloid transfer now give us the opportunity to study the cell and the subcellular factors involved in amyloid deposition.

Honors and Awards: None

Publications:

This work is being prepared for publication and one abstract has already been submitted.

1. Talal, N. and Barth, W. F.: Decreased Ribosome Function During the Induction of Experimental Amyloidosis.

Serial No. NIAMD-ARB-16c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Clinical and Immunological Studies in Sjogren's Syndrome

Previous Serial Number: None

Principal Investigator: Dr. Norman Talal

Other Investigators: Drs. Henry Kaltreider, Elias Zisman (H-CE), Peter Schur, Richard Asofsky (I-LGAR), and Philip Lightbody (D-OMS)

Cooperating Units: National Heart Institute, Clinical Endocrinology Branch; Robert B. Brigham Hospital; National Institute of Allergy and Infectious Diseases, Laboratory of Germ Free Animal Research; National Dental Institute, Oral Medicine and Surgery Branch.

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

To understand the pathogenesis of Sjogren's syndrome and its variants, with particular emphasis on immunologic abnormalities and relationship to lymphoma, pseudolymphoma and renal tubular acidosis.

Methods Employed:

1. Serologic analysis of 70 different Sjogren's sera for six different auto-antibodies.

2. Administration of NH_4Cl to selected patients with measurement of urinary, pH, titratable acidity and ammonium production.

3. Biopsy of minor salivary glands in lower lip for studies of immunoglobulin synthesis (by isotope incorporation and radio-immunoelectrophoresis) and localization (by fluorescence microscopy).

Major Findings:

A. About 20% of patients with Sjogren's syndrome have overt or latent renal tubular acidosis. These patients have hypergammaglobulinemia and a high frequency of auto-antibodies. Based on these findings and on examination of renal biopsies by fluorescence microscopy, we have suggested an auto-immune mechanism for the renal tubular abnormality.

B. Anti-DNA antibodies do not occur in Sjogren's syndrome, perhaps explaining the rarity of glomerulonephritis in comparison to systemic lupus erythematosus.

C. Trigeminal neuropathy has occured in association with peripheral neuropathy in several patients. Three male patients evaluated for possible Sjogren's syndrome have a syndrome of fatty infiltration of the parotid glands associated with obesity, ethanolism and hypertension.

Significance to Bio-Medical Research and the Program of the Institute:

Sjogren's syndrome, in its relationship to hyperglobulinemic purpura, renal disease and malignant lymphoma, is at the corssroads of the autoimmune and neoplastic lymphoid disorders. The elucidation of the renal pathology may shed further light on the autoimmune manifestations of this disease.

Proposed Course:

A. Extension of renal acidification studies to include selected patients with systemic lupus erythematosus, chronic active hepatitis, Waldenstrom's macroglobulinemia with hyperviscosity, and hypergammaglobulinemia of diverse etiology, in a further attempt to elucidate pathogenesis.

B. Study of immunoglobulin synthesis in lip biopsy specimens, and immunoglobulin content in parotid saliva.

Honors and Awards: None

Publications:

1. Talal, N., Sokoloff, L., and Barth, W.: Extra-salivary lymphoid abnormalities in Sjogren's syndrome (Reticulum cell sarcoma, "pseudolymphoma," macroglobulinemia). Amer. J. Med. 43:50-65, 1967.

2. Leventhal, B., Waldorf, D., and Talal, N.: Impaired Lymphocyte transformation and delayed hypersensitivity in Sjogren's syndrome. J. Clin. Invest. 46:1338-1345, 1967.

Serial No. NIAMD-ARB-17c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pathogenesis of Auto-Immunity and Lymphoma in NZB and NZB/NZW F_1 Mice

Previous Serial Number: None

Principal Investigator: Dr. Norman Talal

Other Investigators: Drs. Parker Staples, Peter H. Schur (Robert B. Brigham Hospital), Brigid Leventhal (C-MB), John Sheagren (I-LCI).

Cooperating Units: Robert B. Brigham Hospital; National Cancer Institute, Medicine Branch; National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation.

Man Years:

Total:	1-1/4
Professional:	1
Other:	1/4

Project Description:

Objectives:

The NZB and B/W mice are genetically predisposed to develop Coomb's positive hemolytic anemia, lupus glomerulonephritis, positive LE cells, anti-nuclear factor, and anti-DNA antibodies. In later life, NZB mice also develop malignant lymphomas. These mice represent an excellent laboratory model for studying the pathogenesis of auto-immune disease and its relationship to lymphoid malignancies.

Methods Employed:

A. Induction of immunologic tolerance in young adult mice using ultracentrifuged bovine and human gamma globulin as tolerogens; immunization with antigen in Freund's adjuvant and assay for antibody by immune elimination and hemagglutination.

B. Complement fixation and precipitin assay for serum auto-antibodies (particularly anti-DNA and anti-ribosomes).

C. Reticulo-endothelial clearance of collodial carbon and radioactive aggregated albumin.

D. Response of cultured spleen cells to phytohemagglutinin and DNA.

Major Findings:

A. Tolerance has not been induced in NZB and B/W mice, even to 24 mg of BGG. Tolerance was readily induced in C_3H and C57 black mice, partial tolerance in NZW, but no tolerance in BALC/c mice. NZB and B/W mice also produced greater quantities of antibody to a standard antigenic challenge.

B. There is a difference in the percent incidence of anti-DNA and anti-ribosome (Rb) antibodies in 5 month and older NZB, B/W and NZW mice: NZB (50-60%), B/W (100%), NZW (20%). By contract, the incidence of anti-nucleoprotein antibodies is: NZB (95%), B/W (100%), NZW (66%). This result supports the hypothesis that anti-Rb immune complexes, in addition to anti-DNA, may play a role in the pathogenesis of nephritis in these mice.

C. The clearance of aggregated human albumin is more rapid in NZB than in C_3H mice.

Significance to Bio-Medical Research and the Program of the Institute:

The observation that NZB and B/W mice are relatively resistant to tolerance induction may be related to the lack of self-tolerance that develops later in these animals. This finding could represent a basic defect in immunologic regulation that underlies the development of auto-immune disease and lymphoma.

Proposed Course:

A. Continued attempts to induce tolerance in NZB and B/W mice, using younger animals and more highly purified monomeric BGG.

B. Cell transfer experiments between tolerogenic and atolerogenic mice to define the mechanism of this abnormality.

C. Elution and fluorescent microscopic studies on B/W kidneys to look for anti-ribosome antibodies.

D. Further evaluation of the immunologic capacity and lymphocytic function in NZB and B/W mice.

E. Investigate tolerance induction in C57 black mice neonatally infected with LCM virus, to see whether virus infection can alter the relative ease of tolerance induction.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-18-C
1. Arthritis and Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Metabolic Control of Purine Synthesis and Uric Acid Production in the Normal and Gouty Human Subject.

Previous Serial Number: NIAMD-ARB-16-C

Principal Investigator: Dr. J. Edwin Seegmiller

Other Investigators: Drs. Wilfred Y. Fujimoto, Martin L. Greene, Theodore Friedmann, J. Frank Henderson, Ian C. Caldwell and John H. Subak-Sharpe.

Cooperating Units: University of Alberta, Edmonton, Alberta, Canada; Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry.

Man Years:

Total:	5-1/2
Professional:	3-1/2
Other:	2

Project Description:

Objectives:

Last year we showed that excessive synthesis of uric acid is associated with deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase. Virtually complete absence of the enzyme is associated with an X-linked neurological disease of children and a partial deficiency is found in some patients with typical gouty arthritis with onset in adult life. Elucidation of the mechanism by which the enzyme defect produces excessive purine synthesis should point the way toward detection of other enzyme defects associated with excessive synthesis of uric acid. A new screening test applied to urine for the detection of individuals with this enzyme defect has been evaluated.

Methods Employed:

The mechanism by which this enzyme defect produces excessive purine synthesis has been studied in mutant fibroblasts cultured *in vitro* from simple skin biopsies of affected patients. The rate of purine synthesis in vivo was evaluated by following the incorporation of orally administered glycine-1-^{14}C into urinary uric acid and the turnover of uric acid 1,3-^{15}N administered intravenously as the lithium salt. Purine synthesis in fibroblasts in vitro was assessed by blocking the third step of purine synthesis with azaserine and measuring the incorporation of formate-^{14}C into the formylglycinamide ribonucleotide that accumulates.

The activity of the enzyme HG-PRT was measured by incubating erythrocyte, leukocyte or fibroblast dialyzed lysates with phosphoribosylpyrophosphate (PRPP), magnesium and hypoxanthine-^{14}C or guanine-^{14}C. The ribonucleotides formed in this reaction were separated with carrier nucleotides on paper from the free bases by high voltage electrophoresis, cut out and counted in a liquid scintillation counting system. The activity of a similar enzyme which converts adenine-^{14}C to its ribonucleotide was assayed as a control. The response of different strains of fibroblasts to various inhibitors of purine synthesis was assessed.

The presence of enzyme activity in individual fibroblasts was determined by incubation of the intact cells with a tritiated purine base, washing away the unreacted base. Incorporation of tritiated bases into nucleic acids in normal cells was shown by the dark granules developing with radioautography. Mutant cells failed to incorporate either tritiated hypoxanthine or tritiated guanine into the cells. The interaction of normal and mutant cells grown in the same culture media has been studied by this technique.

As a possible screening test for detection, HG-PRT deficiency was evaluated by determining by automated procedures, both uric acid and creatinine in morning urine samples.

Major Findings:

A four-fold increase in intracellular concentration of PRPP found in mutant fibroblasts lacking the enzyme HG-PRT could contribute to excessive purine synthesis. However, no difference in the intracellular concentration of nucleotides was found.

The demonstration of two cell populations, one with, and one without HG-PRT, in the fibroblasts cultured from the mother of a boy with the complete enzyme defect, provided the first test for the heterozygous carrier of this enzyme defect, as well as additional evidence that this disease is inherited on the X-chromosome.

A total of thirty-six individuals with a deficiency of HG-PRT have been found; twenty with a virtually complete deficiency and the typical neurological symptoms of choreoathetosis, spasticity, mental retardation and compulsive self-mutilation; and sixteen with a partial deficiency and clinical evidence of gouty arthritis and, frequently, uric acid stones of the urinary tract. In addition, six of the latter group had evidence of a relatively mild neurological disease that had not been previously recognized as being associated with the abnormality of purine metabolism. Neurological symptoms were highly variable in different patients and included a mild spastic quadriplegia, mental retardation and seizures.

Evidence of two additional types of enzyme defects have been found in fibroblasts cultured from gouty patients who have normal HG-PRT activity. A markedly diminished response to both natural and synthetic inhibitors of purine synthesis was found in two patients who both produced excessive quantities of uric acid. A third patient showed an accelerated degradation of adenine nucleotides.

HG-PRT has been purified 100-fold from normal human erythrocytes and the kinetics of the normal enzyme has been determined. Evidence has been found that xanthine is also a substrate for HG-PRT.

Studies of the distribution of the HG-PRT show that it is richest in brain, particularly the basal ganglia. A 4- to 5-fold increase over the normal concentration of the oxypurines hypoxanthine and xanthine was found in cerebrospinal fluid of children affected with the complete neurological syndrome. The ratio of uric acid to creatinine in the urine was substantially higher than the normal range in all patients affected with the complete enzyme deficiency, and was outside the normal range in 7 of 8 patients with partial deficiency of HG-PRT.

Significance to Bio-Medical Research and the Program of the Institute:

These studies have helped to define in biochemical terms, a hitherto unrecognized class of neurological dysfunction associated with a partial deficiency of hypoxanthine-guanine phosphoribosyltransferase (HG-PRT) and given some indication of biochemical differences that may mediate the severe neurological dysfunction in children who have the complete deficiency of HG-PRT.

At a basic level these studies suggest that an increased intracellular concentration of PRPP in mutant cells may be one factor contributing to their increased purine synthesis.

The demonstration of the two cell populations in heterozygote carriers of HG-PRT deficiency provides evidence of the validity of the Lyon Hypothesis.

Proposed Course:

Purification of HG-PRT from normal and partially deficient cells will be continued and the physical constants of the proteins will be compared and ultimately their amino acid sequence will be determined. Antibodies prepared against the purified proteins will be used to test for cross-reacting material in cells of patients who have no detectable enzyme activity.

The cells will be used with cells deficient in G6PD, for basic genetic studies of somatic recombination and mapping of the X chromosome. Systems will be devised for the chemical selection of either normal or mutant cells and studies will be made of cellular interaction and possible genetic recombination.

Honors and Awards: None

Publications:

1. Seegmiller, J. E. Intermediary purine metabolism, in Talbott, John H., (Ed.): Gout, New York, Grune and Stratton, 1967.
2. Seegmiller, J. E. Serum Uric Acid, in Cohen, Alan S., (Ed.): Laboratory Diagnostic Procedures in the Rheumatic Diseases, Boston, Mass., Little, Brown & Co., 1967.
3. Seegmiller, J. E. Genetic and molecular basis of human hereditary diseases. Clin. Chem. 13: 554-564, 1967.
4. Kelley, W. N., Rosenbloom, F. M. and Seegmiller, J. E. The effect of azathioprine (Imuran) on purine synthesis in clinical disorders of purine metabolism. J. Clin. Invest. 46: 1518, 1967.
5. Rosenbloom, F. M., Kelley, W. N., Henderson, J. F. and Seegmiller, J. E. Lyon Hypothesis and X-linked disease. Lancet, Aug.5, 305-306, 1967.
6. Kelley, W. N., Rosenbloom, F. M., Henderson, J. F. and Seegmiller, J. E. Xanthine phosphoribosyltransferase in man: Relationship to hypoxanthine-guanine phosphoribosyltransferase. Biochem. & Biophys. Res. Comm. 28: 340, 1967.
7. Rosenbloom, F. M., Kelley, W. N., Miller, J., Henderson, J. F. and Seegmiller, J. E. Inherited disorder of purine metabolism: Correlation between central nervous system dysfunction and biochemical defects. J.A.M.A. 202: 103-106, 1967.

8. Kelley, W. N., Rosenbloom, F. M., Miller, J. and Seegmiller, J. E. An enzymatic basis for variation in response to allopurinol. *New Eng. J. Med.* 278: 287-293, 1968.

9. Seegmiller, J. E., Klinenberg, J. R., Miller, J. and Watts, R. W. E. Suppression of Glycine-N^{15} incorporation into urinary uric acid by Adenine-8-C^{13} in normal and gouty subjects. *J. Clin. Invest.* (May, 1968).

10. Rosenbloom, F. M., Henderson, J. F., Caldwell, I. C., Kelley, W. N. and Seegmiller, J. E. Biochemical bases of accelerated purine biosynthesis *de novo* in human fibroblasts lacking hypoxanthine-guanine phosphoribosyltransferase. *J. Biol. Chem.* 243: 1166-1173, 1968.

11. Seegmiller, J. E. Detection of human inborn errors of metabolism by examination of urinary metabolites. (Symposium on Urinary Constituents of Low Molecular Weight, Oak Ridge, Tennessee, Nov. 30, 1967.) *Clin. Chem.* in press

12. Henderson, J. F., Brox, L. W., Kelley, W. N., Rosenbloom, F. M. and Seegmiller, J. E. Kinetic studies of hypoxanthine-guanine phosphoribosyltransferase. *J. Biol. Chem.* in press.

13. Kaufman, J. M., Greene, M. L. and Seegmiller, J. E. Urine uric acid to creatinine ratio - a screening test for inherited disorders of purine metabolism. *J. Pediat.* in press.

14. Kelley, W. N., Rosenbloom, F. M., Seegmiller, J. E. and Howell, R. R. Excessive production of uric acid in Type I glycogen storage disease. *J. Pediat.* 72: 488-496, 1968.

Serial No. NIAMD-ARB-19-C
1. Arthritis and Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Clinical and Metabolic Evaluation of Cystinosis.

Previous Serial Number: NIAMD-ARB-17-C

Principal Investigator: Dr. J. E. Seegmiller

Other Investigators: Drs. Theodore Friedmann, Jerry A. Schneider, NINDB, and Vernon Wong, NINDB.

Cooperating Units: National Institute of Neurological Disease and Blindness.

Man Years:

Total:	2-1/2
Professional:	1-1/2
Other:	1

Project Description:

Objectives:

We are attempting to define the primary abnormality of metabolism which gives rise to the deposition of cystine crystals in body tissues, characteristic of both the childhood and adult forms of cystinosis. The mechanisms by which the metabolic derangement gives rise to abnormalities of renal tubular function and the progressive glomerular damage leading to death of patients with childhood cystinosis is being investigated. We are also exploring possible methods of treatment of children with this disorder. A possible lead to such treatment is being sought in the definition of biochemical differences in the childhood form of cystinosis, which is invariably fatal, and the adult form of the disease, which seems to be entirely benign.

Methods Employed:

Human fibroblasts cultured *in vitro* from skin biopsies of normal subjects and from patients with both adult and childhood cystinosis and their parents, were used for studies of the intracellular content of cystine and other amino acids, and the incorporation of ^{35}S labeled cysteine and methionine into cysteine and/or cystine of proteins.

A demonstration of the rate of accumulation of intracellular cystine has been attempted using cells incubated with ^{35}S cystine and a disulfide-specific reagent fluorescein mercuric acetate (FMA). We have looked at the integrity of the cysteine synthetic pathway by means of evaluating the conversion of ^{35}S methionine into protein cystine and/or cysteine in cystinotic and normal fibroblasts. In addition we have undertaken some studies on the enzymic degradation of ^{35}S cystine by homogenates of normal cells.

Major Findings:

Fibroblasts obtained from skin biopsies of patients with cystinosis, grown *in vitro*, show marked increases in the concentration of intracellular cystine. The intracellular content of cystine in fibroblasts cultured *in vitro* from patients with childhood cystinosis was over 100 times that of normal fibroblasts. Our finding of around 6 times the normal cystine content in either leukocytes or fibroblasts from their parents, provides the first biochemical abnormality to be found in the heterozygotes for this disease. Leukocytes and fibroblasts from patients with adult cystinosis showed an intracellular cystine content about 40 to 60 times normal. Upon fractionation of the cell, the cystine in both disorders was found in the granular fraction and was liberated into solution by treatment with a detergent. Incubation of normal and cystinotic cells with extensively purified ^{35}S methionine resulted in incorporation of ^{35}S into cysteine moieties of cell protein with the same specific activity in both normal and cystinotic cells. It provides further evidence that the large stores of intracellular cystine are compartmentalized and cannot be utilized for protein synthesis.

Treatment of affected children with penicillamine or with a diet low in sulfur amino acids resulted in a substantial lowering of the plasma cystine content without changing either the cystine content of the leukocytes or the clinical features of the disease.

Significance to Bio-Medical Research and the Program of the Institute:

These findings provide further understanding of the intracellular location of the cystine which accumulates in this disease. The fact that the cystine is compartmentalized and cannot be used by the cells for protein synthesis suggests that attempts at therapy should be directed toward the breakdown of these compartments. The incorporation of ^{35}S from methionine into the cysteine moieties of cell protein show that the cysteine biosynthetic pathway is present in normal and mutant fibroblasts and with the same synthetic rate in both.

The fact that the high cystine content is intracellular suggests that these patients should be as good a candidate for renal transplantation as any other child with renal failure.

Proposed Course:

The precise nature of the primary defect responsible for the high intracellular concentration of cystine will be studied by determining the fate of cystine-^{35}S in the cystine-containing granular fraction of fibroblasts from normal and cystinotic fibroblasts as well as the relative rates of generation and regeneration of SH groups in these cells. The effect of various therapeutic drugs on the intracellular concentration of cystine will be determined.

The effectiveness of renal transplantation in correcting the Fanconi Syndrome of this disease and in changing its clinical course will be determined by cooperative studies with Peter Bent Brigham Hospital in Boston, Massachusetts and Stanford University Hospital in Palo Alto, California.

Honors and Awards: None

Publications:

1. Schneider, J. A., Bradley, K. and Seegmiller, J. E. Increased cystine in leukocytes from individual homozygous and heterozygous for cystinosis. Science 157: 1321, 1967.

2. Crawhall, J. C., Lietman, P. S., Schneider, J. A. and Seegmiller, J. E. Cystinosis: Plasma cystine and cysteine concentrations and the effect of D-penicillamine and dietary treatment. Am. J. Med. 44: 330-339, 1968.

3. Schneider, J. A., Nolan, S. P. and Seegmiller, J. E. Appendicitis in a child with cystinosis. Arch. Surg. in press.

4. Schneider, J. A., Bradley, K. H. and Seegmiller, J. E. Colorimetric assay of cystine using noradrenochrome. J. Analyt. Biochem. 23: 129-133, 1968.

5. Schneider, J. A., Bradley, K. H. and Seegmiller, J. E. Identification and measurement of cysteine-homocysteine mixed disulfide in plasma. J. Lab. & Clin. Med. 71: 122-125, 1968.

6. Schneider, J. A., Rosenbloom, F. M., Bradley, K. H. and Seegmiller, J. E. Increased free-cystine content of fibroblasts cultured from patients with cystinosis. Biochem. Biophys. Res. Comm. 29: 527-531, 1967.

7. Seegmiller, J. E., Friedman, T., Harrison, H. E., Wong, V. and Schneider, J. A. Cystinosis: Combined Clinical Conference, National Institutes of Health, September, 1967. Ann. Int. Med. 68: 883-905, 1968.

Serial No. NIAMD-ARB-20-C
1. Arthritis and Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1967 through June 30, 1968

Project Title: Metabolic Factors Causing a Renal Retention of Uric Acid and Hyperuricemia.

Previous Serial Number: NIAMD-ARB-18-C

Principal Investigator: Dr. J. Edwin Seegmiller

Other Investigators: None

Cooperating Units: None

This project is to be continued but time did not permit further work during this period.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-21-C
1. Arthritis and Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of Crystals of Monosodium Urate in the Genesis of Gouty Arthritis and a Mechanism for the Therapeutic Action of Colchicine.

Previous Serial No. NIAMD-ARB-19-C

Principal Investigator: Dr. J. E. Seegmiller

Other Investigators: None

Cooperating Units: None

This project is being continued but has been interrupted for lack of suitable patient material for study.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-22-C
1. Arthritis and Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemical Basis of Maple Syrup Urine Disease

Previous Serial Number: NIAMD-ARB-21-C

Principal Investigator: Dr. J. Edwin Seegmiller

Other Investigators: Drs. Roland G. Westall and Charles E. Dent

Cooperating Units: University College Hospital, London, England

This project has been interrupted because we do not have access to suitable patients.

Honors and Awards: None

Publications:

1. Seegmiller, J. E. and Westall, R. G. The enzyme defect in maple syrup urine disease (branched chain ketonuria). J. Mental Deficiency Res. II: 288-294, 1967.

Serial No. NIAMD-ARB-23-C
1. Arthritis and Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Metabolic Actions of Salicylic Acid and Aspirin

Previous Serial Number: Same

Principal Investigator: Dr. Peter Goldman

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: -1/4
Other: -3/4

Project Description:

Objectives:

To study the metabolic alterations which are induced by salicylates.

Methods Employed:

Tissues removed at surgery or from experimental animals are being used to study certain metabolic reactions and the effect of salicylate on these reactions. The tissues are used as minces, homogenates or partially purified enzymes, depending on the metabolic pathways and the tissues under study. The incorporation of radioactive precursor into product has been used as a measure of the metabolic pathway.

In addition, an examination is being made of the chemical properties of salicylate and some of its analogues with a view toward establishing correlations that may explain mode of action.

Major Findings:

Salicylate has been found to inhibit the biosynthesis of long-chain fatty acids in cell free systems from rat liver and adipose tissue. This effect is due to the inhibitory action of salicylates on the enzyme acetyl-CoA carboxylase. Since this enzyme has also been shown to be involved in cholesterol biosynthesis, these studies could explain the therapeutic action of salicylate in lowering blood cholesterol as well as the finding that salicylates inhibit long-chain fatty acid synthesis in vivo.

Significance to Bio-Medical Research and the Program of the Institute:

Salicylates are used for a variety of analgesic functions in addition to being a major therapeutic agent in rheumatoid arthritis. Their mode of action is largely unknown. Recently studies have raised the question of whether these compounds are teratogens. The lack of knowledge about these drugs which are used widely and over long periods of time suggests the need for further studies on them at a biochemical level.

Proposed Course:

The chemical and biochemical effects of salicylates will be studied in human and animal systems.

Honors and Awards: None

Publications:

None

Serial No. NIAMD-ARB-24-C
1. Arthritis and Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Characteristics of the Carbon-Halogen Bond in Compounds of Biological Importance.

Previous Serial Number: Same

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. George W. A. Milne (H-LM)

Cooperating Units: National Heart Institute
Laboratory of Metabolism

Man Years:

Total:	1
Professional:	-1/2
Other:	-1/2

Project Description:

Objectives:

To elucidate the enzyme mechanisms involved in the cleavage of the carbon-halogen bond.

Methods Employed:

By the technique of enrichment culture, microbes have been isolated which can utilize as a sole carbon source, compounds containing the carbon-halogen bond. Such organisms contain enzymes which can cleave the carbon-halogen bond and attempts have been made to characterize these enzymes. In addition, some of these organisms are able to form various halogenated intermediates which have been isolated and characterized.

Major Findings:

Studies on the mechanism of carbon-fluorine bond cleavage in the enzyme catalyzing the reaction $H_2O + FCH_2COO^- \rightarrow HF + HOCH_2COO^-$ have shown that this reaction is not reversible. In addition, by the use of H_2O^{18} it has been shown that the oxygen in the hydroxyl group of glycolate is derived from water rather than from reduced oxygen as in the carbon-fluorine bond cleavage catalyzed by several mixed-function oxidases. The reactivity of this enzyme named haloacetate halidohydrolase toward other halogenated analogues of fluoroacetate has been studied. The results indicate that in this enzyme the relative reaction rates for dehalogenation are F > Cl > I. This is in an order exactly opposite to that which might be predicted from the bond energies involved in these reactions.

To explore further the relative reactivities of other enzymes toward such a class of halogenated compounds a microbe has been isolated which can utilize dichloroacetate as a sole source of carbon. Two halidohydrolases have been isolated from this organism, one induced by chloroacetate, the other by dichloroacetate. These enzymes differ in various properties including size and heat stability. Though each attacks the same substrates with the same K_m, the enzymes differ in their relative rates of reaction with the various substrates. These substrates include L-2 chlorobutyrate, L-2 chloropropionate, monochloroacetate, dichloroacetate and iodoacetate. The stereospecificity of the substrates and the inversion of configuration which accompanies the hydrolytic cleavage provide additional insight into the mechanism of this class of reaction.

Significance to Bio-Medical Research and the Program of the Institute:

The similarity in size between the carbon-fluorine bond and the carbon-hydrogen bond provides the basis of the action of two important metabolic inhibitors, e.g., 5-fluorouracil and fluorocitrate. The introduction of the carbon-fluorine bond also changes the reactivity of certain steroids, e.g., 9-α-fluorohydrocortisone. The stability of the carbon fluorine bond in most living systems prevents degradation of such compounds and thus increases their usefulness as drugs and metabolic inhibitors. On the other hand the carbon-iodine bond is extremely labile and because of this it has been hard to distinguish enzymatic and non-enzymatic reactions. It is hoped that by understanding the mechanism of carbon-halogen reactions in general, one may understand the biological reactions which break the carbon-iodine bond in some of the thyroid hormones.

Proposed Course:

A number of compounds containing the carbon-halogen bond will be used to isolate organisms which contain enzymes which degrade these compounds. These enzymes will be studied to elucidate the mechanisms of fission of the carbon-halogen bond. When more is learned of the mechanisms and methodology involved, some analogous enzymes in mammalian systems can be examined. In addition, those enzymes which may be particularly suited to the delineation of enzyme mechanisms will be studied in more detail.

Honors and Awards: None

Publications:

1. Goldman, P., Milne, G. W. A. and Keister, D. B. Carbon-Halogen Bond Cleavage III: Studies on bacterial halidohydrolases. J. Biol. Chem. 243: 428, 1968.

Serial No. NIAMD-ARE-25-C
1. Arthritis and Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Microbial Metabolism of Drugs which are Analogues of Normal Biochemical Intermediates.

Previous Serial Number: Same

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Carl C. Levy (C-D)

Cooperating Units: Dermatology Branch
National Cancer Institute

Man Years:

Total:	-1/2
Professional:	-1/4
Other:	-1/4

Project Description:

Objectives:

To isolate organisms which can utilize certain drugs as sole sources of carbon and/or nitrogen in order to study the metabolic pathways, and the enzymes involved.

Methods Employed:

By the technique of enrichment culture, microbes have been isolated which can utilize drugs as sole sources of carbon and/or nitrogen. Such organisms contain enzymes which metabolize these compounds. These enzymes and the metabolic products of the drugs are characterized.

Major Findings:

A pseudomonad has been found which can utilize methotrexate as a sole source of carbon and nitrogen. An enzyme from this organism has been found which degrades methotrexate according to the equation Methotrexate ⟶ 4-amino-4-deoxy-10 methyl pteroic acid + glutamate. This enzyme has been purified and shown to catalyze a similar reaction toward folate and aminopterin. The spectral changes which accompany this reaction have formed a basis for a specific enzymatic assay of folate and its analogues in biological fluids such as urine.

When the pseudomonad is grown on folic acid instead of methotrexate, pteroic acid is excreted stoichiometrically into the growth medium. This observation has been exploited to provide a method for the preparation of pteroat from the readily available and inexpensive folate. Pteroic acid, synthesized by a tedius organic synthesis is no longer available from the drug companies, and this method provides a simple preparation for the biochemist and the pharmacologist.

The enzyme which removes glutamate from the folate analogues has been found to be a peptidase, specific for the removal of glutamate from a wide variety of compounds, with a terminal glutamate. The enzyme, now called carboxypeptidase G, has an absolute requirement for Zn^{++} and because of its unique specificity may provide an interesting model system for this class of reactions.

The study with carboxypeptidase G has provided a model for the selection of new peptidases specific for a particular amino acid. Using the folate analogue 4-amino-4-deoxypteroylaspartate, an organism has been found which has an enzyme or enzyme-specific for the N-acyl linuages to aspartate or asparagine. In this system there may be two enzymes, one specifically requiring Co^{++}, the other requiring Zn^{++}.

A third enzyme has been found which removes alanine from 4-amino-4-deoxypteroylalanine. It is interesting that among these three enzymes, each is specific for the hydrolysis of the analogue containing the particular amino acid.

Significance to Bio-Medical Research and the Program of the Institute:

Methotrexate is widely used in the chemotherapy of cancer and in several other diseases. Biological transformation of this drug has been found in man, rabbit, and in certain bacteria. However, the products of the metabolism have not been characterized. By the methods described it is possible to elucidate metabolic pathways of these drugs in biological systems. Certain of the enzymes or microbes involved in these studies can provide the basis for specific drug assays where no such assays exist. Such assays might assist in the development of optimal chemotherapeutic uses of these drugs.

Proposed Course:

This method for the isolation of specific peptidases will be exploited to find other enzymes of this class. Those enzymes which have properties suggesting that they might provide useful information on peptidase mechanisms will be studied further. In addition, these enzymes will be used as tools to delineate biochemical properties of folate analogues.

Honors and Awards: None

Publications:

1. Levy, C. C. and Goldman, P. The Enzymatic Hydrolysis of Methotrexate and Folate. J. Biol. Chem. 242: 2933, 1967.

2. Goldman, P. and Levy, C. C. Carboxypeptidase G: Purification and Properties. Proc. Nat. Acad. Sci. 58: 1299, 1967.

3. Levy, C. C. and Goldman, P. Bacterial Peptidases II: Enzymes specific for N-acyl linuages to aspartate. J. Biol. Chem. in press.

4. Goldman, P. and Levy, C. C. Bacterial propagation of pteroic acid. (Biochemical Preparations, 1969) in press.

METABOLIC DISEASES BRANCH

The three autonomous sections of MDB have continued their respective lines of investigation during FY 1968 without major shifts in emphasis. There was no change in the permanent staff pattern, although the usual biennial changes in associate staff occurred. The growing stature of the Section on Gastroenterology was evidenced by the arrival of two guest workers - Dr. Claude Perrier from Geneva, Switzerland, and Dr. Albert Dibbins from Pittsburgh, Pennsylvania.

Section on Physiology and Clinical Nutrition

Mechanisms of Secretion in Human Eccrine Sweat Glands: We continue to be fascinated by the fact that these minute glands can transport outward such relatively large volumes of secretion; relative to size their fluid transport capacity can be rivaled only by renal tubules. However, there appear to be major metabolic differences, the most evident of which is the extremely high rate of glycolysis manifested by sweat gland tissue. Lactate production is vigorous *in vivo*, as is shown by the high lactate content of sweat, and in isolated tissue *in vitro*, which produces extraordinary amounts of lactate per unit of tissue weight and time. Isolated sweat gland tissue *in vitro* would appear to be suitable for further physiologic and biochemical studies, as it retains ultrastructural integrity for some time, and responds to physiologic stimuli by an increased output of lactate.

(Drs. S. Wolfe and R. Gordon)

Sweat rate in normal volunteers has been followed carefully by frequent weighings while in a warm, humid environment. The rate has been found to be influenced markedly by hydration, and sweat rate will increase sharply within 2 minutes after the subject drinks water at body temperature. We are currently studying the mechanism of this apparent reflex.

(Drs. S. Wolfe, R. Thompson, and R. Gordon, and Mrs. H. Miller)

The chemical composition of eccrine sweat collected on filter paper discs applied to sweating skin can be studied in relation to sweat rate. A large volume of such data was collected during FY 1966 and 1967, and is now being analyzed and reduced to manageable proportions with the assistance of computer programs. We have derived an empirical equation which related sweat potassium concentration to sweat rate for each individual, and can then compare the parameters of this equation with other factors, most importantly serum potassium. The correlation of sweat with serum potassium is highly significant at all but the lowest sweat rates; this probably means that the hypothetical precursor fluid secreted by the distal portion of the sweat gland contains potassium in equilibrium with serum potassium, and that extra potassium enters the sweat in the duct. At very low sweat rates, the contribution of the duct is predominant, but at higher rates sweat potassium levels reflect the concentration in the precursor fluid.

(Drs. G. Cage, S. Wolfe, and R. Gordon)

Dietary Effects on Whole Body Potassium: An earlier study, undertaken to find out whether heat stress and sweating influenced overall potassium balance significantly, led to observations on the effect of a diet composed mostly of rice on body potassium content. The whole body counter showed potassium content of normal volunteers to fall by up to 15%, but this tendency to fall could not be fully corrected by increasing the potassium intake. We formulated the hypothesis that another dietary factor, most likely magnesium, was inadequate in the rice diet, even though standard nutritional tables had indicated that the diet was adequate.

This question has been re-opened with new volunteers, who have taken the rice diet for periods up to six weeks, in the absence of any unusual heat stress. The first two groups have been studied, although the analysis of data is not complete at this writing. It appears that there is, in fact, a noticeable decrease in potassium content on the diet alone, but that the provision of supplements of both potassium and magnesium salts will prevent this. By continuing such studies for many months with many normals, we hope to define the threshold level of magnesium intake which will effectively prevent loss of body potassium.

(Drs. R. Gordon and S. Wolfe)

Total Energy and Substrate Metabolism: The NIAMD metabolic chamber, which dates from the initiation of clinical research at NIH, was originally designed to measure total energy turnover in human subjects (both normal and diseased) by indirect calorimetry. Both the instrumentation of the chamber and its principles had to a certain extent been superseded by scientific advances of the past decades, most particularly by the introduction and widespread utilization of isotopic techniques both for the diagnosis of thyroid dysfunctions, and in the study of the conversion of energy-yielding substrates to CO_2 in the intact human subject. However, the chamber still offered an excellent environment for the study of energy metabolism, as it provides for the precise control of environmental thermal stress, and offers a place apart from the inevitable traffic in a medical ward, where a single subject can be studied extensively, and gas exchange as well as other physiologic parameters measured and recorded. It was therefore determined that the gas exchange instrumentation should be revised, utilizing newer and more accurate devices, and allowing for the simultaneous determinations of levels of $^{14}CO_2$ in expired air.

FY 1968 has seen design work completed, a contract let, and a large fraction of the new instrumentation fabricated. Full installation and testing are scheduled for the next few months. A late innovation was provision for data presentation on punched tape as well as in writing, so that when computer processing of output data is desirable, it will be possible to avoid the intermediate steps of transcribing information at a keypunch machine.

(Drs. R. Thompson and R. Gordon, and Mr. D. Thrasher)

Opportunistic Studies of Unusual Patients: Two unusual clinical situations which presented themselves during FY 1967 and 1968 have been exploited without benefit of advanced planning. In one case, a preoperative diagnosis of Meckel's diverticulum was made in spite of the fact that standard investigative studies failed to reveal it. This was accomplished by passing a tube, fabricated for the purpose, to the location of the diverticulum, and instilling a small amount of a thin suspension of barium, followed by an x-ray. The lesion was removed, the patient cured, and the case is being prepared for publication as a report.

The other situation was presented by two siblings with an apparently hereditary disorder characterized by failure to sweat, failure to perceive pain, mental retardation, and destructive bone and joint changes. Although the basic etiology has not been elucidated, this syndrome has been studied in detail and a manuscript reporting our findings is in preparation.

(Drs. S. Wolfe and R. Gordon, and colleagues from several other Institutes)

Section on Gastroenterology

Studies of the small intestine:

1. Gluten-sensitive enteropathy. We have measured the capacity of the mucosa of the human small intestine to synthesize protein *in vitro* by determining incorporation of L-leucine-U-^{14}C in the total protein. Patients with flattened intestinal mucosa tended to have a rate of synthesis approximately twice normal, whereas patients with congenital beta-lipoprotein deficiency had a rate of about one-third normal. Specimens from patients with active granulomatous enteritis and treated Whipple's disease in remission were either only slightly elevated or within the normal range. As an extension of this study and as a major finding, we determined the effect of gliadin on the *in vitro* assay. Gliadin is the alcohol-soluble fraction of wheat gluten, the protein responsible for the intestinal abnormalities in patients with gluten-sensitive enteropathy. Gliadin *stimulated* mucosal protein synthesis by specimens from a) normal volunteers, b) a patient with flattened intestinal mucosa who was not sensitive to dietary gluten, c) a patient with tropical sprue in remission, d) two patients with treated Whipple's disease, and e) two patients with granulomatous enteritis. In striking contrast gliadin *inhibited* protein synthesis by specimens from patients with flattened intestinal mucosa who were sensitive to dietary gluten. The difference in response was highly significant ($p < 0.001$). We believe this observation constitutes the first demonstration of a biochemical difference in responsiveness between mucosa from patients with gluten-sensitive enteropathy and from normal subjects or patients with other intestinal diseases. This observation may prove useful in studying the pathophysiology of gluten toxicity, as well as in the rapid diagnosis of gluten sensitivity.

(Drs. Andrew L. Warshaw and Leonard Laster, NIAMD)

2. Studies of the catabolism of the C-terminal tetrapeptide of gastrin *in vitro*. Gastrin is a single polypeptide chain comprising 17 amino acids. The C-terminal tetrapeptide possesses the full range of physiological activity of the entire molecule, although in reduced potency. When the amide group is missing from the C-terminal tetrapeptide the molecule is physiologically inactive. We explored the mammalian inactivation of this polypeptide hormone fragment by incubating tissue extracts with the tetrapeptide. We detected in various rat tissues a deamidase activity which catalyzes the removal of the terminal amide group. A quantitative method for measuring this enzymatic activity was developed with BOC-Try-Met-Asp-Phe-amide as the substrate. Appropriate conditions for the assay were determined and the generation of equivalent amounts of the deamidated tetrapeptide free acid and of ammonia was demonstrated. The enzyme is particularly active in liver and small intestine mucosa. It has been obtained in soluble form, and its purification is now under study. This enzyme may represent an important means of deactivating the hormone *in vivo*.

(Drs. John H. Walsh, NCDC, and Leonard Laster, NIAMD)

3. Application of biophysical techniques to the study of the small intestine. A new method has been developed for the preparation of biological tissues for examination under the electron probe microanalyzer. This method permits the localization of elements within specific regions of tissue cells, and with this method we have performed a preliminary study of intestinal goblet cells. Striking variations in potassium, chloride, sulfur, and phosphorus content from other areas of the cell were detected.

(Drs. A. J. Tousimis, Biodynamics Research Corp., and Leonard Laster, NIAMD)

Inborn errors of metabolism

1. Hypophosphatasia. It has generally been assumed that in hypophosphatasia all tissues in which alkaline phosphatase activity normally occurs are deficient in that enzyme. We examined the question of whether the deficiency affects the mucosa of the small intestine. We assayed biopsy specimens from normal volunteers and from members of a kindred with hypophosphatasia for intestinal alkaline phosphatase activity with four different substrates. Contrary to expectations there was no deficiency of intestinal alkaline phosphatase activity detected in any of the patients with hypophosphatasia. In a few instances the activity was abnormally increased.

(Drs. Stuart H. Danovitch, NIAMD, Paul N. Baer, NIDH, and Leonard Laster, NIAMD)

2. Sulfite oxidase deficiency. To explore further some of the questions arising from our discovery of the inborn error of metabolism, sulfite oxidase deficiency (described in the last report), we are examining the question whether normal man excretes S-sulfo-L-cysteine in his urine. We have preliminary evidence to suggest that he does, and we are now isolating the material from normal urine in order to characterize it.

(Drs. Filadelfo Irreverre, NIAMD, S. Harvey Mudd, NCI, William D. Heizer and Leonard Laster, NIAMD, and Alan K. Percy, NINDB)

Section on Mineral Metabolism

Investigations are continuing utilizing metabolic balance techniques and calcium-47 kinetic analyses to determine the mechanism whereby phosphate and other dietary factors cause increased calcium storage, diminished urinary calcium excretion and reduced incidence of renal colic. Observations to date suggest that the effect of phosphate is manifested through promotion of bone formation rates. The mechanism for this effect is at the moment unknown.

(Drs. Melson, Chase, Whedon and Aurbach)

During the past year studies on the structure, regulation of secretion, and mechanism of action of parathyroid hormone have progressed towards solution of a number of the problems involved. Application of the Edman reaction has allowed deduction of the amino acid sequence of a major portion of the polypeptide fragment that represents the active portion of the parathyroid hormone molecule. The area of the molecule requisite for biological and immunological activity is represented by a sequence approximately 30 amino acids in length at the amino-terminal part of the polypeptide chain.

(Dr. Aurbach (NIAMD), Drs. Keutmann, Niall, Deftos, Brewer and Potts (NHI))

Further studies have been carried out to determine physiologically important factors regulating the secretion of parathyroid hormone. Application of a sensitive radioimmunoassay for the hormone showed that parathyroid hormone secretion increases as blood calcium falls and decreases as blood calcium rises; blood phosphate does not influence parathyroid hormone secretion. Analysis by computer showed a highly significant direct inverse linear relationship between hormone secretion and blood calcium concentration. It was concluded that calcium regulates parathyroid hormone secretion by a proportional control mechanism.

(Drs. Aurbach (NIAMD), Buckle, Deftos and Potts (NHI))

Human parathyroid hormone has been isolated from adenomas taken from patients with hyperparathyroidism. The human hormone shows reduced affinity for antisera directed against bovine hormone; it has been concluded therefore that the human and bovine hormones are immunologically distinct. Physical and chemical studies including amino acid analyses show the human hormone to be quite similar to the bovine hormone with the exception of two to three amino acids. Presumably these small but significant differences account for the differences in immunological reactivity of the two molecules.

(Drs. O'Riordan and Aurbach (NIAMD), and Dr. Potts (NHI))

Studies during the past year have led to the identification of the probable mechanism of action of parathyroid hormone. It was found that the urinary excretion of cyclic 3'5'-AMP is influenced to a large extent by the rate of secretion of parathyroid hormone. In addition, injection of exogenous hormone causes a marked and rapid rise in the excretion of cyclic AMP into the urine. These studies suggested that parathyroid hormone action is mediated through activation of adenyl cyclase, the enzyme converting ATP to cyclic AMP in the kidney. Subsequent tests have shown that parathyroid hormone *in vitro* directly activates adenyl cyclase in the kidney as well as in bone, the two organs known to be the physiologic sites for parathyroid hormone activity. In addition, studies in our laboratory showed that the adenyl cyclase of the kidney can be separated into two anatomical zones; adenyl cyclase in the cortex responds virtually exclusively to parathyroid hormone. The cyclase system in the medulla responds primarily to vasopressin.

(Drs. Chase, Melson and Aurbach)

Studies on pseudohypoparathyroidism: metabolic abnormality underlying the disorder. The finding that parathyroid hormone action is likely mediated through activation of adenyl cyclase led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3'5'-AMP in the urine. Radioimmunoassay of parathyroid hormone was carried out as described previously. Approximately 15 of the 70 cases described in the literature have been examined to date. We have found virtually without exception that subjects with pseudohypoparathyroidism do not respond to parathyroid hormone with the normal rise in excretion of cyclic AMP in the urine. We have also found that immunologically detectable parathyroid hormone exists in high concentrations in the circulation of these subjects and that this high rate of secretion of hormone can be inhibited by hypercalcemia as is the case with normal subjects. Even when endogenous parathyroid hormone secretion was suppressed, exogenous hormone caused no change in excretion of cyclic AMP. This experiment tended to rule out the possibility that an abnormal hormone was secreted in this disorder which was biologically inert yet capable of interfering with the action of exogenous parathyroid hormone. These several findings have led to the conclusion that the cause of this disorder may be the existence of genetically deficient or defective adenyl cyclase in the bone and kidney of these subjects.

(Drs. Chase, Melson and Aurbach)

A study was made to determine the physiological mechanism whereby thyrocalcitonin causes hypocalcemia. Calcium-45 was given intravenously to test rats before administering thyrocalcitonin. The hypocalcemia induced by thyrocalcitonin was occasioned by a rise and plateau in the specific activity of blood calcium. These observed changes were reproduced in a computer model system when the return of unlabeled calcium from a reservoir was switched off. The most likely location of this reservoir is bone, and it is apparent that thyrocalcitonin acts by suppressing resorption of bone calcium.

(Drs. O'Riordan and Aurbach)

Serial No. NIAMD-MDB-1 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in Bone Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. G. L. Melson, Lewis Chase and G. D. Whedon

Cooperating Units: Dr. Mones Berman, Mathematical Research Branch, NIAMD
Dr. James Phang, NCI

Man Years:
Total : 2-5/8
Professional: 3/8
Other : 2-1/4

Project Description:

Objectives: 1) To investigate the factors influencing mineral storage and loss in demineralizing bone diseases.

2) To investigate the response of the parathyroid gland to rapid changes in blood calcium.

3) To study the influence of phosphate on resorption of bone mineral.

Methods Employed: 1) Metabolic balance studies in patients with disorders of calcium metabolism, noting the effects on nitrogen, calcium, phosphate and magnesium balances of various dietary intakes of calcium, phosphate and magnesium.

2) Estimation of pool sizes, turnover rates, bone deposition rates, and absorption rates of calcium in patients by the oral and intravenous administration of tracer doses of Ca^{47}.

Major Findings: 1) Effects of feeding phosphate. High phosphate intakes have been found to improve calcium balance through reduction of urinary calcium loss. Preliminary observations suggest that the effect of phosphate is manifested through a diminution of bone resorption rate. Several patients with urethral lithiasis appear to have greatly reduced incidence of renal colic once the phosphate feeding therapy was instituted.

Honors and Awards: None

Publications:

1. Birge, S. J., Jr., and Whedon, G. D.: Bone. In McCally, M. (Ed.): Hypodynamics and Hypogravics. New York, N. Y., Academic Press, Inc., in press.
2. Whedon, G. D.: Osteoporosis. In Astwood, E. B., and Cassidy, C.E. (Eds.): Clinical Endocrinology II. New York, Grune & Stratton, 1968, pp. 349-376.

Serial No. NIAMD-MDB-2 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of Parathyroid Hormone: Physiological Regulation of Secretion; Physical, Chemical, and Immunochemical Properties; Biochemistry; and Structure, and Mechanism of Action.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Dr. J. L. H. O'Riordan, Dr. H. T. Keutmann, Dr. John T. Potts, Jr., Dr. Hugh Niall, Dr. Lewis Chase, Dr. Richard Buckle, Dr. Leonard Deftos, and Dr. Brian Brewer.

Cooperating Units: NHI, Laboratory of Metabolism
University of Pennsylvania School of Veterinary Medicine

Man Years:
Total : 4-7/8
Professional: 1-1/2
Other : 3-3/8

Objectives: To prepare purified parathyroid hormone in pilot-plant quantities; to study chemical properties and structure of the hormone; to relate chemical properties and structure to biological activity and to immunochemical behavior; to examine parathyroid-sensitive systems *in vitro* with a view toward elucidating the mechanism of action of the hormone; to develop a clinically useful test for circulating parathyroid hormone; to study the physiological regulation of the secretion of the hormone.

Methods Employed: Highly purified hormone is prepared from phenol extracts of bovine parathyroid glands by solvent fractionation gel filtration and chromatography on carboxymethylcellulose. Purity is assessed by disc gel electrophoresis, ultracentrifugation, immunodiffusion and immunoelectrophoresis, and recovery of the amino- and carboxyl-terminal amino acids. The hormone is assayed *in vivo* in rats or by a highly sensitive radioimmunoassay. Specific chemical and enzymatic methods are used to cleave the hormone to smaller polypeptide fragments. The polypeptide derivatives are sequentially degraded with exopeptidases and the Edman reaction. Methods have been developed to measure intracellular concentrations of certain enzymes and substrates including cyclic AMP. Tissues, particularly kidney, are tested for enzymatic activity and substrate content during periods immediately following injection of the hormone _in vivo_.

Major Findings: Chemistry: The amino- and COOH-terminal residues have been identified as alanine and glutamine respectively. Each peptide produced from the hormonal molecule by digestion with trypsin has been analyzed for amino acid composition. Analyses of other peptide fragments produced by chemical or enzymatic means and analyses of exopeptidase digests of the hormone yielded sufficient information to assign each "tryptic" peptide to a position in the molecule; this assemblage of peptide subunits thus placed in linear order has become a working model for the structure of the hormonal molecule. The absolute sequence for the major fraction of the first 35 amino acid residues has been deduced. An extremely sensitive method (Pisano and Niall, NHI) for detecting phenylthiohydantoin derivatives of amino acids using gas chromatography was utilized in this work. The method also allows for detection of impurities in hormone preparations.

Structure-Function Studies on Parathyroid Hormone: Certain areas in the molecule necessary for biological and immunological activity were identified by assaying subunits produced chemically or by enzymatic digestion. It has been known for years that the biological activity of the hormone is stable to heating in weak hydrochloric acid; this weak acid treatment produces partially active, smaller fragments of the hormone. Analysis of one particular fragment showed that it represented a minimal area of the molecule requisite for biological and immunological activity. This fragment accounts for a sequence approximately 20 amino acids long. A slightly smaller segment in the same area proved to be important for immunological activity. Further studies on enzymatically and chemically modified hormone preparations showed that the methionine, tryptophan and tyrosine within the acid fragment region are each critically important for biological activity; modification of the methionine and tryptophan does not severely alter immunological reactivity but the latter is destroyed by oxidizing the tyrosine in the molecule.

Physiology: Further studies have been carried out to determine physiologically important factors regulating the secretion of parathyroid hormone. Studies were carried out to measure secretion rates in cows (with Dr. G. T. Mayer at the University of Pennsylvania), sheep and goats (with Dr. A. D. Care) making use of the highly sensitive radioimmunoassay for parathyroid hormone developed in collaboration with Drs. S. Berson and R. Yalow at the Bronx V. A. Hospital. Intravenous infusions of calcium induced a sharp fall in the concentration of parathyroid hormone in blood; conversely infusions of EDTA led to a sharp increase in circulating hormone. Experiments with perfused isolated parathyroid glands showed that the changes in peripheral concentration of hormone actually reflected the true secretion rate. High concentrations of magnesium also inhibit hormone secretion. Other experiments with infusion of phosphate showed that it does not significantly affect the secretion of parathyroid hormone. It was concluded that blood calcium is the principal determinant of parathyroid hormone secretion.

Mechanism of Action: A highly sensitive and specific test for 3'5'-AMP was developed based on enzymatic conversion of the cyclic nucleotide to ATP and measurement of the latter via the ^{32}Pi-ATP exchange reaction catalyzed by the coupled triosephosphate dehydrogenase-phosphoglycerate kinase enzyme system. This system detects cyclic AMP in quantities ranging from 0.006 to 0.25 nMoles. A highly specific method for isolating cyclic AMP from urine was devised based on barium zinc fractionation. The barium zinc supernatant was passed over a small Dowex-50 column and lyophilized. The lyophilized residue was then tested for cyclic AMP. A number of studies have already been carried out showing that the parathyroid status of the rat is the most important factor regulating excretion of cyclic AMP in the urine. Calcium infusions into normal rats caused an immediate decrease in the rate of excretion of cyclic AMP; a similar effect was produced by parathyroidectomy. Conversely, injection of small amounts of parathyroid hormone intravenously causes a marked and rapid increase in the excretion of cyclic AMP in the urine. This effect is seen earlier than the well-known phosphaturic response to the hormone, the latter being one of the earliest *in vivo* responses to the hormone heretofore described.

The above effects suggested that the mechanism of action of parathyroid hormone is mediated through activation of adenyl cyclase. Subsequent studies have shown that parathyroid hormone *in vitro* activates adenyl cyclase in the kidney as well as bone, the two organs known to be the physiologic sites for parathyroid hormone activity. As with other hormone systems mediated through activation of adenyl cyclase, the stimulation of this enzyme in kidney and bone is virtually immediate. In addition, studies in our laboratory showed that the adenyl cyclase of kidney can be separated into two anatomical zones; adenyl cyclase in the cortex responds virtually exclusively to parathyroid hormone. The cyclase system in the medulla responds primarily to vasopressin. Further studies show that virtually all adenyl cyclase detectable in kidney tissue is found within the renal tubules. Again, renal tubular elements from the cortex respond primarily to parathyroid hormone, while those in the medulla respond to vasopressin. This work establishes virtually beyond doubt that the receptor for parathyroid hormone in the kidney resides within the renal tubule as has been suspected but not proven by indirect *in vivo* physiological experiments.

Human Parathyroid Hormone: The properties of human parathyroid hormone (HPTH) have been studied. Work in this field has been handicapped by the limited supply of material (100 G of adenomat contained about 1 mg of hormone). To make it feasible to monitor the extraction process, the radioimmunoassay of bovine parathyroid hormone (BPTH) has been modified for rapid detection of 4 - 20 mμg (for bioassay 100 μg would have been necessary). It has been shown that HPTH behaves like BPTH on extraction with urea and acetic acid and on salt fractionation. It is precipitable with TCA. On

Sephadex G-100 it has a partition coefficient similar to that of bovine parathyroid hormone (BPTH) and it chromatographs on carboxymethylcellulose at pH and ionic strength similar to that needed for BPTH. Studies on the purified product by disc gel electrophoresis show that approximately 200 micrograms of the human hormone had been isolated in apparently pure form.

With Dr. P. G. Condliffe, the behavior of HPTH in the ultracentrifuge has been examined. This has been possible by combining sucrose gradient centrifugation at speeds higher than previously possible with the sensitive immunoassay method. It has been shown that sedimentation coefficients for HPTH and BPTH are the same.

Despite these similarities, the question remains whether HPTH has sufficient affinity for anti-bovine antisera to allow the use of BPTH as standard for the immunoassay of HPTH in the serum of patients. If similar affinities were assumed, it would seem that the concentration of parathyroid hormone in man is lower than in the blood of several other species. If the antibody has a lower affinity (as seems more likely from studies on the small amount of material isolated in purified form) for HPTH, then the concentration would be underestimated. HPTH has been labeled with ^{131}I in order to analyze its behavior in the radioimmunoassay.

Significance to Bio-Medical Research and Program of the Institute: Nothing is known about the nature of circulating parathyroid hormone in metabolic diseases of bone or in clinical disturbances of parathyroid function. These general problems can only be resolved by gaining basic knowledge of the structure and physico-chemical properties of parathyroid hormone.

Proposed Course of Project: The "tryptic" peptides of parathyroid hormone are now being isolated on large scale; as each of these subunits is isolated, it will be degraded further to determine the internal sequences within particular areas of the hormonal molecule. Particular effort is being expended to isolate the active 20 amino acid fragment in high yield so that its entire internal sequence can be determined. Once this is known it would be possible to synthesize a molecule having parathyroid hormone activity. The entire investigation will undoubtedly lead to the complete amino acid sequence of the native molecule. Studies on the mechanism of action of this molecule are continuing. The results found for the effect of hormone on cyclic AMP lead to the conclusion that parathyroid hormone is yet another polypeptide whose mechanism of action is mediated intracellularly via the cyclic nucleotide. These findings show further that the hormone activates adenyl cyclase, the membrane-found enzyme catalyzing conversion of ATP to 3'5'-AMP, in the hormonally-sensitive tissues kidney and bone. It may be possible to isolate the adenyl cyclase sensitive to parathyroid hormone in the kidney. It is expected that the radioimmunoassay will develop into a useful test in the diagnosis of clinical disorders and will continue to yield important information concerning immunoreactive sites in the hormonal polypeptide molecule.

Honors and Awards: None

Publications:

1. Aurbach, G. D., and Potts, J. T., Jr.: The chemistry of parathyroid hormone. In Leonardi, A., and Walsh, J. (Eds.): Proceedings of the First International Symposium on Drugs of Animal Origin. Milan, Ferro Edizioni, 1967, pp. 147-155.
2. Chase, L. R., and Aurbach, G. D.: Parathyroid function and the renal excretion of 3'5'-adenylic acid. Proc. Nat. Acad. Sci. USA 58: 518-525, Aug. 1967.
3. O'Riordan, J. L. H., Condliffe, P. G., Potts, J. T., Jr., and Aurbach, G. D.: Density gradient centrifugation of human and bovine parathyroid hormone. Endocrinology 81: 585-590, Sept. 1967.
4. Ramberg, C. F., Mayer, G. P., Kronfeld, D. S., Aurbach, G. D., Sherwood, L. M., and Potts, J. T., Jr.: Plasma calcium and parathyroid hormone responses to EDTA infusion in the cow. Am. J. Physiol. 213: 878-882, Oct. 1967.
5. Aurbach, G. D., and Potts, J. T., Jr.: The chemistry of parathyroid hormone. In Leonardi, A., and Walsh, J. (Eds.): Proceedings of the First International Symposium on Drugs of Animal Origin. Milan, Ferro Edizioni, 1967, pp. 147-155.
6. Deftos, L. J., Rabson, A. S., Buckle, R. M., Aurbach, G. D., and Potts, J. T., Jr.: Parathyroid hormone production in vitro by human parathyroid cells transformed by Simian virus 40. Science 159: 435-436, Jan. 1968.
7. Chase, L. R., and Aurbach, G. D.: Renal adenyl cyclase: anatomically separate sites for parathyroid hormone and vasopressin. Science 159: 545-547, Feb. 1968.
8. Chase, L. R., and Aurbach, G. D.: Cyclic AMP and the mechanism of action of parathyroid hormone. In Talmage, R. V., and Belanger, L. (Eds.): Proceedings of the Third Conference on Parathyroid Hormone and Thyrocalcitonin. Amsterdam, The Netherlands, Excerpta Medica Foundation, 1968, pp. 220-229, in press.
9. Aurbach, G. D., Sherwood, L. M., and Potts, J. T., Jr.: Regulation of parathyroid gland function. In Astwood, E. B., and Cassidy, C. E. (Eds.): Clinical Endocrinology II. New York, Grune & Stratton, 1968, pp. 324-335.
10. O'Riordan, J. L. H., Potts, J. T., Jr., and Aurbach, G. D.: Characterization of human parathyroid hormone. Proceedings of the Vth European Symposium on Calcified Tissues, 1968, in press.

11. Buckle, R. M., Aurbach, G. D., and Potts, J. T., Jr.: The use of radioimmunoassay of parathyroid hormone in the assessment of adaptation of parathyroid glandular activity. In Margoulies, M. (Ed.): Proceedings of the International Symposium on Protein and Polypeptide Hormones. Amsterdam, Excerpta Medica Foundation, 1968, pp. 389-395.

Serial No. NIAMD-MDB-3 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Chemical Nature and Mode of Action of Thyrocalcitonin.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Dr. Lewis Chase, Dr. G. L. Melson, Dr. J. L. H. O'Riordan, Dr. James Phang.

Cooperating Units: Office of Mathematical Research, NIAMD
National Cancer Institute
Department of Pharmacology, University of North Carolina

Man Years:
Total : 7/8
Professional: 1/2
Other : 3/8

Project Description:

Objectives: To study the chemical nature of thyrocalcitonin and the mechanism whereby this factor lowers blood calcium and induces net increase in bone formation.

Methods Employed: Separation methods involving gel filtration, column electrophoresis, chromatography and high speed density gradient ultracentrifugation. Kinetic analysis made use of the distribution of calcium-45 injected intravenously into rats or calcium-47 injected intravenously into human subjects. The effect of thyrocalcitonin, a factor inducing hypocalcemia, is examined via a kinetic model set up with the computer program developed by Dr. Mones Berman, Office of Mathematical Research.

Major Findings: In collaboration with Drs. Tashjian, Munson and Condliffe, members of the Mineral MetabolismSection determined that the molecular weight of thyrocalcitonin is in the range of 5000, which is considerably less than what is heretofore recognized. This was determined by density gradient ultracentrifugation. A molecular weight almost twice this has been proposed by other workers based on their study of an impure preparation of the material.

Mode of Action: In rats it has been found that thyrocalcitonin causes a slight rise and plateau in the specific activity of blood calcium in animals given calcium-45 acutely before the test period. The observed changes were reproduced in the computer model system when the return of unlabeled calcium from a reservoir switched off. The most likely location of this reservoir is bone, and it is apparent that thyrocalcitonin acts by suppressing bone resorption.

A relatively large dose of thyrocalcitonin (16 MRC units) was injected intravenously into a human subject who had previously been studied with calcium kinetic analysis during a control period. The intravenous dose of thyrocalcitonin caused a slight fall in serum calcium (a decrement of 0.4 mg/100 ml) which, however, was significant. Analyses of the data are being carried out to determine whether the action of thyrocalcitonin in human subjects is via a similar method as was found with rats.

Significance to Bio-Medical Research and Program of the Institute: Since thyrocalcitonin inhibits resorption of calcium from bone, it is possible that this factor would have potential therapeutic importance in certain clinical disorders of bone demineralization.

Proposed Course of Project: Further studies are planned to determine whether thyrocalcitonin can be a useful agent in diminishing bone resorption in certain abnormal disease states. In particular, it will be of interest to study the effect of thyrocalcitonin in hypervitaminosis D, hyperparathyroidism, and certain malignant states that produce an abnormally high rate of resorption of bone with hypercalcemia. Further chemical studies are in progress to define more closely the chemical nature of thyrocalcitonin.

Honors and Awards: None

Publications:

1. O'Riordan, J. L. H., and Aurbach, G. D.: The effect of thyrocalcitonin on calcium exchange. *Pharmacologist* 9: 72-73, 1967.
2. O'Riordan, J. L. H., and Aurbach, G. D.: Mode of action of thyrocalcitonin. *Endocrinology* 82: 377-383, Feb. 1968.

Serial No. NIAMD-MDB-4 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Pseudohypoparathyroidism: Metabolic Abnormality Underlying the Disorder.

Previous Serial Number: NONE

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Lewis Chase, G. L. Melson, J. T. Potts, Jr., Leonard Deftos, and Richard Reitz.

Cooperating Units: NHI, Laboratory of Metabolism
Massachusetts General Hospital Endocrinology Service

Man Years:
Total : 1-3/4
Professional: 5/8
Other : 1-1/8

Project Description:

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our findings that parathyroid hormone action is likely mediated through activation of adenyl cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3'5'-AMP in the urine.

Methods: Parathyroid hormone purified in our laboratories was sterile-filtered for clinical use through courtesy of the NIH Pharmacy. Radioimmunoassay of parathyroid hormone was carried out as described previously. Measurement of 3'5'-AMP was performed according to the procedure devised in this laboratory.

Major Findings: Approximately 70 cases of this disorder have been described in the literature; we have examined 13 of these to date. In normal subjects, parathyroid hormone causes a 30- to 60-fold rise in urinary excretion of cyclic AMP within 30 minutes or less after intravenous administration of the hormone. A similar response has been observed in idiopathic, hypoparathyroid, surgical hypoparathyroid, and pseudopseudohypoparathyroid subjects (the latter category show the constitutional changes but do not have laboratory evidence of hypoparathyroidism). Parathyroid hormone caused no increase in excretion of cyclic AMP in patients with pseudohypoparathyroidism. In one case, studied through the cooperation of a clinical investigator at another hospital, there was a moderate rise in excretion of cyclic AMP; however, the diagnosis in this instance may be incorrect, and as yet we have not had the opportunity of seeing the patient at the Clinical Center. Patients with this disorder who fail to respond to parathyroid hormone showed a similarly defective response after prolonged calcium infusion. Parathyroid hormone was detectable in the plasma of pseudohypoparathyroid subjects before infusion of calcium but not after. This experiment tended to rule out the possibility that an abnormal endogenously secreted hormone immunologically reactive but biologically inert interfered with the action of exogenous parathyroid hormone.

These findings have led to the conclusion that the test for urinary cyclic AMP after parathyroid administration is a better diagnostic index of pseudohypoparathyroidism than measurements of phosphate clearance used heretofore. In addition, it appears likely that the cause/of this disorder may be the existence of genetically deficient or defective adenyl cyclase in the bone and kidney of these subjects.

Significance to Bio-Medical Research and Program of the Institute: These studies appear to resolve a long-standing controversy concerning the nature and cause of the disease, pseudohypoparathyroidism. Our findings suggest that this may be another disease attributable to a genetically aberrant enzyme, and show for the first time that the parathyroid hormone endogenously secreted in this disorder is still under the control of calcium in the extracellular fluid. In addition, this "experiment of nature" provides further support for the hypothesis that the mechanism of action of parathyroid hormone is mediated through activation of adenyl cyclase in the specific target organs responsive to parathyroid hormone.

Proposed Course of the Project: Additional patients are being sought in order to extend these findings further. It is hoped that opportunity may arise to obtain biopsy samples of bone and kidney from one or more of these subjects; this would allow conclusive studies on the sensitivity of adenyl cyclase in this disorder to parathyroid hormone. The tests we have developed should be helpful in delineating the genetic mechanisms underlying transmission of this disease.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-5 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Total Energy and Substrate Metabolism: Studies in Health and Disease

Previous Serial Number: NIAMD-MDB-4 (c)

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigator: Dr. Ronald H. Thompson

Cooperating Units: None

Man Years:
Total : 1
Professional: 1/2
Other : 1/2

Project Description:

Objectives: To apply the technique of continuous, extended indirect calorimetry (respiratory gas exchange) and a number of related metabolic and physiologic techniques to the clinical study of energy metabolism, a) to elucidate the mechanisms of normal physiologic response, particularly temperature regulation, to various stimuli including climatic stress, b) to study the influence and mechanism of action on metabolism of a variety of endocrine hormones and drugs, and c) to investigate the characteristics of energy expenditure, energy balance, and aberrations of metabolic processes in a variety of disease conditions.

Methods Employed: Indirect human calorimetry by means of complete continuous expired air analysis in the Metabolic Chamber, metabolic balance determinations, and total body heat loss by thermoelectric and radiometric techniques.

Major Findings: This project is expected to continue as a long-range interest of NIAMD, MDB. During FY '68 plans for reinstrumentation of the metabolic chamber were completed, and a contract negotiated and signed for the production and installation of the necessary equipment. Parts of the new instrumentation have been built by the personnel of the laboratory. The old equipment has been dismantled and removed, with those components which can be reutilized being salvaged.

During this period, clinical studies of gas exchange have been suspended.

Significance to Bio-medical Research and the Program of the Institute: Studies of human energy metabolism in the metabolic chamber are concerned with a variety of basic problems of physiology and metabolism and are thus directly related to the principal interests of NIAMD research. The unique facility (Chamber) employed in these studies permits continuous and precise measurement of total energy expenditure, and of the effects of various hormones on energy metabolism.

Proposed Course of Project: It is anticipated that some six months will be required to complete the installation and testing of the new equipment. We expect to include provisions for the output of data by teletype and punched tape, so that results can be immediately entered into computers for further processing when required.

These improvements should make it possible to undertake a much wider range of metabolic studies, which will include the rates of conversion of various labeled substrates to CO_2 as well as total energy turnover. These techniques will then be applied to the study of as many patients as pose problems amenable to this approach. Of particular interest should be studies of the conversion of labeled substrates to CO_2 in subjects with various inborn errors of metabolism.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-6 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of Diet on Potassium Balance

Previous Serial Number: NIAMD-MDB-5 (c)

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators: Drs. Sidney M. Wolfe and Ronald H. Thompson

Cooperating Units: The Whole Body Counter Unit, Department of Nuclear Medicine, NIH
NCI, Metabolism

Man Years:
Total : 1
Professional: 1/8
Other : 7/8

Project Description:

Objectives: This project is an outgrowth of the previous study undertaken to learn whether or not significant losses of body potassium would occur in volunteers living on a diet composed primarily of rice (like the diets eaten in Southeast Asia) and exposed to environmental heat stress. Studies on normal volunteers were completed during FY '66, with the finding that losses of up to 15% of body potassium content would occur within a few months. It was also demonstrated that such losses did not occur in volunteers on the standard house diet. Supplementation of the rice diet with pure potassium salts tended to ameliorate, but did not prevent, the loss of body potassium. It was therefore supposed that the diet was deficient in some other nutrient, most likely magnesium. Similar studies are now being taken up once again, omitting the element of heat stress, but incorporating controlled variations of magnesium intake.

Methods: Normal volunteers are receiving the diet, some with and some without supplements of potassium salts, magnesium salts, or both. Whole body potassium, body weight, body volume (with the assistance of Dr. N. Berlin, NCI,Metabolism), and blood and urine chemistries are being followed. Data are being recorded on IBM cards for subsequent processing and graphing by computer.

Major Findings: Until a sufficient number of volunteers have been studied so that comparisons can be made, no conclusions are possible. However, it is already clear that the rice diet alone, without the added potassium drain of sweating, will produce a significant fall in body potassium content. Thus this diet, which was at first considered to satisfy all known human nutritional requirements, is inadequate in some respect. Since the diet corresponds in most details with the overall composition of diets reported in several surveys of Southeast Asian countries, the definitive identification of the deficiency is of considerable interest.

Significance to Bio-medical Research and Program of the Institute: There now seems little question that a rice diet containing a high content of salt, but presumably adequate amounts of other nutrients, whether or not combined with significant sweating, produces a decrease in body potassium content. The precise cause of this potassium loss is not known, but the phenomenon demands attention. Further definition of the human dietary requirement for magnesium will clearly be of great importance.

Proposed Course of Project: Enough volunteers must be studied to allow comparison of effects at different levels of magnesium intake. The data will be plotted and compared with the help of various computer programs now being written and tested.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-7 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanisms of Secretion in Human Eccrine Sweat Glands

Previous Serial Number: NIAMD-MDB-6 (c)

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators: Dr. Sidney M. Wolfe, Dr. Ronald H. Thompson and Dr. Gary W. Cage

Cooperating Units: None

Man Years:
Total : 2-1/2
Professional: 1-3/8
Other : 1-1/8

Project Description:

Objectives: The eccrine sweat gland of man is an unusual structure, and has few parallels in lower animals. Human sweat glands are numerous, but small; their aggregate mass in the average man is on the order of only 30 grams. Nevertheless, this tissue is capable of producing secretion at rates exceeding 30 cc/min. At lower sweat rates, there are marked differences between the chemical composition of sweat and the composition of the interstitial fluid from which it is derived. Even at higher sweat rates, where most electrolyte concentrations are very close to those of extracellular fluid, the secretion contains very little protein or glucose, only traces of amino acids, and large amounts of lactate. Thus, it is apparent that an active secretory process of very high capacity is present in these minute glands.

An understanding of the chemistry of normal sweat secretion could be of great value in understanding and possibly in correcting the abnormalities in a number of diseases. In the case of cystic fibrosis, where it is presumed that a single inherited molecular defect is responsible for the many varied clinical manifestations of disease, the sweat gland may well be that tissue in which the basic biochemical defect can be most easily investigated.

Methods and Major Findings: Sweat gland function has been studied in two principal ways. The tissue may be investigated in situ, functioning under the normal physiologic stimulus of heat, or it may be isolated by microdissection from surviving skin in vitro. Under the latter circumstances, one is free to analyze the tissue, subject it to a wide variety of conditions that could not be realized in vivo, and use radioisotopes in doses that could not be used on the living subject.

Investigations in vivo have continued along the lines outlined last year. Normal volunteers have been made to sweat in the environmental chamber, and the sweat has been collected for chemical analysis on discs of filter paper. This allows for the simultaneous determination of sweat composition and sweat rate; it is clear that the chemistry of sweat is markedly influenced by the rate of its secretion. A large mass of such data has been collected, and is now being coded and analyzed by computer programs. Empirical equations which give a good fit to the relationship of sweat solute concentration to sweat rate have been derived, and we are now investigating the relationship of the parameters of these equations to other factors, such as blood chemistry. Preliminary results appear to show that there is a highly significant correlation between sweat potassium and serum potassium, and the regression equation appears to show a direct relationship with a slope of 1. This would imply that sweat potassium is derived directly from serum, without any major modification which might be adaptive or tending to preserve homeostasis.

By weighing our sweating subjects at frequent intervals, we can follow their overall rate of water loss with considerable accuracy. The installation of a Brookline metabolic scale in the environmental chamber will make this measurement simpler and even more precise. Such serial weighings have been used to show a marked difference in sweat rate between a subject when losing moisture rapidly, and the same individual when hydration is preserved by generous intake of water p. o. We have found that the rapid ingestion of 1 to 2 liters of water at body temperature will produce a rapid increase in sweat rate in a subject who has been allowed to dehydrate himself; this increase may exceed 50% of the previous rate, and may develop in as little as 2 minutes. Studies to discover the mechanism of this reflex are under way. It seems difficult to believe that so much water has actually been absorbed within so short a time, yet it is equally difficult to envision an alternative reflex mechanism that would so rapidly and effectively signal to the sweat glands that an increased rate of sweating can be tolerated.

These findings with respect to the interrelationship of hydration and sweat rate have led us to discount the effects earlier observed when acetazolamide was found to diminish sweat rate in normal volunteers. The diuretic effect of this drug would have led to relative dehydration, and the decreased sweat rate was probably only a secondary effect.

Isolated sweat gland tissue, analyzed in vitro, has been tested for its content of glycogen and of carbonic anhydrase. The latter appears to be virtually absent, while glycogen is abundant. Sweat glands incubated in vitro consume their glycogen and produce lactate, and have so far not been shown to use appreciable amounts of oxygen. Thus anaerobic glycolysis appears to be the principal metabolic energy source in this tissue. Glycolysis is stimulated by epinephrine and by acetyl-beta-methyl choline, and the stimulatory effect of the latter can be blocked by atropine. These findings suggest that the isolated tissue in vitro is functioning, and responding to stimuli, in a relatively normal way.

Significance to Bio-medical Research and the Program of the Institute: An understanding of the secretory mechanisms of the sweat gland may well lead to better understanding and control of specific disorders of this tissue. It may also throw light on more general problems related to the transport of water and solids by epithelia, and could facilitate the discovery of the basic molecular defect in cystic fibrosis of the pancreas.

Proposed Course of Project: In the coming year, we shall carry out a number of studies designed to uncover the mechanism of the reflex effect of hydration on sweat rate. Subjects will ingest water containing a dilute suspension of barium, and we will carry out x-ray studies to determine whether or not the water has left the stomach when its effect on sweating has occurred. We shall also attempt to block this effect with food and other materials which might delay gastric emptying. A new investigation of the effect, if any, of antidiuretic hormone on sweat gland function may be in order.

We shall also study the relationship of sweat rate to sweat solute concentration with and without hydration, as this may show whether the hydration effect is due to recruitment of additional sweat glands, or to a greater sweat output per gland.

With the acquisition of a liquid scintillation counter, we should be able considerably to expand the study of sweat gland metabolism in vitro. A first study will be to learn whether or not the tissue will produce $^{14}CO_2$ from labeled substrates. Another lead which may be exploited is our earlier observation that stimulation of the gland in vitro will increase the incorporation of ^{32}P into phospholipids, as has already been shown for so many other secretory tissues. These phenomena may be utilized indirectly to study the dependence of the sweat gland on oxygen in the environment, on electrolyte content of the medium, and on other factors.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-8 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of an Unusual Syndrome in Two Siblings

Previous Serial Number: NIAMD-MDB-7 (c)

Principal Investigator: Dr. Sidney M. Wolfe

Other Investigators: None

Cooperating Units: Laboratory of Clinical Investigation, NIAID, Cardiology Branch and Clinical Endocrinology Branch, NHI

Man Years:
Total : 1/8
Professional: 1/8
Other : 0

Project Description:

This limited project was described in some detail last year. Briefly, two siblings with an unusual disorder characterized by poikilothermia, diminished sensibility to pain, virtual anhydrosis, and mental retardation have been studied in detail. Their syndrome does not coincide precisely with any recognized disorder, such as familial dysautonomia, and should probably be considered as a new disease.

Significance to Bio-medical Research and Program of the Institute: Abnormalities encountered in these children may lead to new insights into normal aspects of temperature regulation. Of course, one always hopes that better physiologic understanding of a disease state such as this will lead to improved methods of prevention or treatment.

Proposed Course of Project: The clinical studies having been completed, the results of the investigation of these two children are being prepared for publication. Unless other individuals with the same disease are referred to the Clinical Center, the study will of necessity be terminated.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-9 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: An Elegant Method of Preoperative Diagnosis of Meckel's Diverticulum

Previous Serial Number: None

Principal Investigator: Dr. Sidney M. Wolfe

Other Investigators: Dr. Robert S. Gordon, Jr., Dr. John D. Harrah, Dr. William R. Merritt, and Dr. Robert C. Hoye

Cooperative Units: NCI Surgery
CC Radiology

Man Years:
Total : 1/8
Professional: 1/8
Other : 0

Project Description:

This ad hoc project was initiated when a young man, admitted to the Clinical Center as a normal volunteer for other studies, was found to have chronic intermittent GI bleeding and an iron deficiency anemia. Routine clinical workup suggested the diagnosis (by exclusion) of Meckel's diverticulum, but it proved to be impossible to visualize the lesion radiographically by routine techniques. Rather than recommend an explortory operation on negative evidence, we determined to attempt to localize and identify the lesion by a novel technique.

An intestinal tube was prepared from PE-190 polyethylene tubing, a finger cot, mercury, and silk thread. The subject swallowed this, and as it progressed slowly through the GI tract, material was aspirated and tested for blood. When positive results were finally obtained, the location of the tube was documented both by its length and by x-ray. A thin suspension of barium was then instilled just at the point of presumed blood loss, and films outlined a typical Meckel's diverticulum. With the preoperative diagnosis thus established, curative surgery was successfully undertaken.

A case report is being prepared for publication.

Significance to Bio-medical Research and Program of the Institute: Clearly, a definite preoperative diagnosis is of considerable value when major surgery is being contemplated. This study shows that selective opacification of the GI tract may yield x-ray data not available by standard techniques. Such tests may eventually become routine as part of the study that precedes laparotomy in cases where the diagnosis is difficult, and where the situation is not so urgent that surgery cannot safely be delayed.

Proposed Course of Project: We should be happy to have another occasion to try this procedure, but will not be able to do so unless another such subject comes to our attention. Young people with Meckel's diverticulum are not ordinarily referred to the Clinical Center, as definitive treatment is usually completed elsewhere.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-10c
1. Metabolic Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of the small intestine

Previous Serial Number: NIAMD-MDB-8c

Principal Investigator: Dr. Leonard Laster

Other Investigators: Drs. Paul N. Baer, NIDR, Stuart H. Danovitch and William D. Heizer, NIAMD, Thomas A. Waldmann, NCI, Andrew L. Warshaw, NIAMD, and Elliot Weser, formerly NIAMD.

Cooperating Units: National Cancer Institute
National Institute of Dental Research

Man Years:

Total: 3
Professional: 1
Others: 2

Project Description:

Objectives:

The purpose of this study is to investigate 1) intermediary metabolism of the mucosa of the small intestine, 2) factors which regulate mucosal metabolism, and 3) effects of disease on these metabolic processes.

Methods Employed:

Various techniques for studying tissue metabolism were employed. These included the use of isotopically labeled compounds *in vitro* and *in vivo*, and methods pertinent to enzymology.

Patient Material:

For the studies of protein synthesis, biopsy specimens were taken from 15 normal volunteers and from approximately 25 patients with various diseases of the small intestine. For the study of hypophosphatasia, some 8 family members of a kindred with the disease were admitted for study, and approximately 15 volunteer controls were used in addition. For the investigation of protein-losing gastroenteropathy in association with laxative ingestion, 2 patients were studied.

Major Findings:

1. Protein synthesis in the human small intestine mucosa (Drs. Andrew L. Warshaw and Leonard Laster, NIAMD)

Biopsy specimens of mucosa of human small intestine were incubated *in vitro* with L-leucine-U-^{14}C and incorporation of isotope into total protein was determined. Appropriate conditions for this assay were determined, and used to establish the mean and range of values for mucosal protein synthesis in 15 normal volunteers. With 7 patients having flattened intestinal mucosa the mean was approximately twice normal; with two patients having congenital betalipoprotein deficiency the mean was about one-third normal; in two patients with active granulomatous enteritis the mean was slightly higher than normal; and in 5 patients with treated Whipple's disease in remission the mean was normal. These findings indicate that various diseases have a consistent and definite effect on protein synthesis by intestinal mucosa.

In the second part of the study, the effect of the presence of gliadin on mucosal protein synthesis *in vitro* was evaluated. Gliadin is the alcohol-soluble fraction of the protein gluten of wheat, and is responsible for the intestinal abnormalities in patients with gluten-sensitive enteropathy. The addition of gliadin resulted in *stimulation* of protein synthesis by biopsy specimens from 11 normal volunteers (+24%), one patient with flattened intestinal mucosa who was not sensitive to dietary gluten (+10%), one patient with tropical sprue in remission (+27%), two patients with treated Whipple's disease (+34%), and two patients with granulomatous enteritis (+70%). In sharp contrast, addition of gliadin *in vitro* *inhibited* protein synthesis by biopsy specimens from patients with flattened intestinal mucosa who were sensitive to dietary gluten (-28%). We believe this observation constitutes the first demonstration of a biochemical difference in responsiveness between mucosa from patients with gluten--sensitive enteropathy and normal subjects or patients with other intestinal diseases. This finding may be useful in studying the pathophysiology of gluten toxicity, in testing fractions of gliadin for toxicity in order to determine the specific peptide structure responsible for the injurious effects, and perhaps in the rapid diagnosis of gluten sensitivity.

2. Intestinal alkaline phosphatase activity in familial hypophosphatasia (Stuart H. Danovitch, NIAMD, Paul N. Baer, NIDR, and Leonard Laster, NIAMD)

Hypophosphatasia is a genetically determined metabolic disease characterized by three types of phenotypic expression. Type I starts *in utero* or within the first six months of life, and is associated with severe skeletal abnormalities, hypercalcemia, occasionally renal insufficiency, and usually death during the first year. Type II, a less severe form, is associated with clinical abnormalities which gradually become apparent after the age of 6 months, and which include rechitic skeletal changes and premature loss of the primary teeth. Type III is usually asymptomatic, but may be associated with osteoporosis. The disease is characterized chemically by a reduction in the concentration of alkaline phosphatase in the serum and by abnormally increased urinary excretion of phosphorylethanolamine. It has generally been assumed that in hypophosphatasia all tissues in which alkaline phosphatase is normally found show a reduction in this enzymatic activity. However, the studies showing the reduction in the alkaline phosphatase activity of the small intestine were carried out with tissues obtained postmortem, and even in those studies there was some question whether the reduction in intestinal activity was as severe as that in bone. To determine directly whether or not hypophosphatasia affects small intestine alkaline phosphatase activity we obtained peroral biopsy specimens of intestinal mucosa from members of two kindred with types II and III hypophosphatasia and assayed the specimens for alkaline phosphatase activity with each of the following substrates: disodium phenyl phosphate, phosphorylethanolamine, phosphoryl choline, and myo-inositol-2-phosphate. Standard conditions for assays of human intestinal mucosa were determined. For each subject, the abnormal urinary excretion of phosphorylethanolamine was documented by chemical determinations, and as a measure of the viability of the mucosal specimens, intestinal disaccharidase activity was also determined. Contrary to what we anticipated, we found that intestinal alkaline phosphatase activity in the subjects with hypophosphatasia was either normal or abnormally elevated. In no case was it reduced. Since it must be assumed that the low serum alkaline phosphatase activity in these patients reflected a reduction in alkaline phosphatase activity in some tissue of the body, probably bone and possibly liver, the finding of normal intestinal phosphatase activity raises the question of whether alkaline phosphatase activity in different tissues is under different genetic control mechanisms.

3. Metabolism of D-xylose and D-xylonic acid *in vivo* and *in vitro* by the guinea pig (Elliot Weser, formerly NIAMD, and Leonard Laster, NIAMD)

In previous reports we summarized studies of the oxidation of D-xylose and D-xylonic acid *in vivo* and *in vitro* by tissues of the guinea pig. A description of this work is to be published this year.

4. Protein-losing enteropathy (Drs. W. D. Heizer and A. L. Warshaw, NIAMD, Thomas A. Waldmann, NCI, and Leonard Laster, NIAMD)

In our previous report we noted a study of two patients who, because of emotional disturbances, ingested markedly excessive quantities of laxatives. Each patient suffered a reduction in serum albumin concentration as a consequence of the drug ingestion, and it was shown that the reduction in serum protein was associated with excessive gastroenterological loss of proteins. Withdrawal of the laxatives reversed the protein-losing enteropathy. These studies were published during the past year, and a number of gastroenterologists have informed us that reading the paper led them to establish a diagnosis in patients who had represented long-standing diagnostic problems.

Significance to Bio-medical Research and the Program of NIAMD:

These studies provide insight into the pathophysiology of gluten--sensitive enteropathy and point the way to further exploration of that disease. They also provide information about the effects of hypophosphatasia and cathartics in abnormal doses on the small intestine mucosa.

Proposed Course:

These general projects will be continued and the findings reported here will be extended.

Honors and Awards:

The studies of protein synthesis by human intestinal mucosa (Warshaw and Laster) were selected for presentation at the Scientific Session of the Annual Meeting of the American Society for Clinical Investigation, Atlantic City, May 6, 1968. The studies of intestinal alkaline phosphatase activity in hypophosphatasia (Danovitch, Baer and Laster) were selected for presentation at the Metabolism Sub-specialty Section Meeting of the American Federation for Clinical Research, Atlantic City, May 5, 1968.

Publications:

Danovitch, S. H., Baer, P. N., and Laster, L.: Intestinal alkaline phosphatase activity in familial hypophosphatasia. New Engl. J. Med. In press.

Weser, E., and Laster, L.: Metabolism of D-xylose and D-xylonic acid *in vivo* and *in vitro* by the guinea pig. Biochem. Med. In press.

Heizer, W. D., Warshaw, A. L., Waldmann, T. A., and Laster, L.: Protein-losing gastroenteropathy and malabsorption associated with factitious diarrhea. Ann. Intern. Med. 68: 839-852, April 1968.

Serial No. NIAMD-MDB-11c
1. Metabolic Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Whipple's disease and on the small intestine in general

Previous Serial Number: NIAMD-MDB-9c

Principal Investigator: Dr. Leonard Laster

Other Investigators: Drs. Peter M. Loeb, NIAMD, A. J. Tousimis, Biodynamics Research Corp., and Leonard Laster, NIAMD

Cooperating Units: Biodynamics Research Corp. (Contract #PH 43-66-10)

Man Years:

Total: 2
Professional: 1
Others: 1

Project Description:

Objectives:

This is a long-term project designed to evaluate the pathologic physiology of Whipple's disease and to study the effect of therapy on its natural history. This investigation was described in previous reports (see NIAMD-MDB-9c, 1966-67).

Methods:

The general approach to this study includes serial assessment of intestinal absorption and protein exudation during the course of the disease; examinations of intestinal morphology by light and electron microscopy; and the use of electron probe microanalysis to study intestinal tissues.

Patient Material

For the study of Whipple's disease, the various patients we have been following were admitted from time to time for reevaluation by assessment of intestinal function and by studies of intestinal biopsies. For the study of the tight junction of the human intestinal epithelium, specimens taken from various normal volunteers were used.

Major Findings:

1. Clinical (Drs. Peter M. Loeb and Leonard Laster, NIAMD)

The long term study on Whipple's disease continues. We are now following six patients in remission. Some have been off therapy for at least five years, and appear to be doing well. In time this material will be collected in a monograph and be presented.

2. Biophysical approaches to the study of human disease (Drs. A. J. Tousimis, Biodynamics Research Corp., and Leonard Laster, NIAMD)

A collaborative study has been developed with Dr. A. J. Tousimis for the purpose of applying biophysical techniques to the study of biological problems. At the moment the small intestine is being used as a model tissue. The techniques involved include high resolution electron microscopy and electron probe microanalysis. This work is sponsored under NIH Contract #PH 43-66-10 with Biodynamics Research Corporation ($69,450 for FY 1968). This contract is now reaching the end of its third year, and an application for its renewal will be submitted.

A. Electron probe microanalysis.

Studies of uptake of L-selenomethionine by Amphiuma small intestine reported last year had to be modified because it became apparent that the available methods for the examination of biological tissues by electron probe microanalysis were unsatisfactory. Accordingly, the past year was devoted to the development of a new method for processing tissues. It allows for much more precise localization of elements within tissue structures. With this method we have been exploring the goblet cell of the Amphiuma small intestine and have found it to be comparatively rich in sulfur, potassium and chloride, and low in phosphorus. With this new methodology now available we are exploring various tissues of the Amphiuma for their elemental content.

B. Studies of the intestinal mucosa.

Although it has been generally assumed that in the region of tight junction between epithelial cells, the outer leaflets of the plasma membranes of the two cells are fused, our examination of biopsy specimens from human intestinal mucosa suggests that these membranes may not be fused but merely in close apposition, and in some cases overlying one another. A detailed study of the electron microscopic appearance of the tight junction of the human intestinal mucosa has been completed and the results are now being readied for publication.

Significance to Bio-medical Research and the Program of NIAMD:

These studies are designed to provide insight into the structure and chemical composition of intestinal mucosa in health and disease.

Proposed Course:

If support for Contract #PH 43-66-10 is continued, all of these studies will be developed further.

Honors and Awards:

The studies of electron probe microanalysis were presented by invitation at the Eastern Analytical Symposium on Microanalysis in New York City, November 10, 1967.

Publications:

None.

Serial No. NIAMD-MDB-12c
1. Metabolic Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biochemical aspects of disease

Previous Serial Number: NIAMD-MDB-10c

Principal Investigator: Dr. Leonard Laster

Other Investigators: Drs. William D. Heizer and Filadelfo Irreverre, NIAMD, S. Harvey Mudd, NIMH, Alan K. Percy, NINDB, Joseph Volpe, NICHD, John B. Walsh, NCDC, and Andrew L. Warshaw, NIAMD.

Cooperating Units:
National Communicable Disease Center
National Institute of Child Health & Human Development
National Institute of Mental Health
National Institute of Neurological Diseases and Blindness.

Man Years:

Total: 2
Professional: 1
Others: 1

Project Description:

Objectives:

The purpose of this study is to examine pathways of metabolism in tissues of the gastrointestinal tract and the disruption of these pathways in disease.

Methods Employed:

In vitro studies included procedures commonly employed in enzymology and were used for identification and assay of the various catalytic activities studied. In the clinical studies, assays of intestinal absorption, and measurements of parathyroid and pancreatic function were made.

Patient Material:

For the studies of inborn errors, 3 control children were used for urine collections. For the study of pancreatic insufficiency and hyperparathyroidism, 1 patient was used.

Major Findings:

1. Inborn errors of the metabolism of sulfur-containing amino acids (Drs. Filadelfo Irreverre, NIAMD, S. Harvey Mudd, NIMH, William D. Heizer and Leonard Laster, NIAMD, and Alan K. Percy, NINDB)

In our last report we described our discovery of a previously unrecognized disease characterized by mental retardation, dislocated ocular lenses, and abnormal urinary excretion of S-sulfo-L-cysteine. We obtained evidence that the disorder was attributable to a deficiency of sulfite oxidase activity, and the enzymatic defect was then demonstrated directly in tissues obtained from the patient after his death. These studies have now been described in detail in a publication.

Since last year, we have extended this work to include a study of various sulfated compounds in the patient's urine and tissues. We reasoned that a deficiency of sulfite oxidase should have caused a deficiency of sulfate, which in turn might have impaired production of such sulfate-containing materials as myelin in brain and tyrosine-O--sulfate in urine. We assayed specimens from control children and from the patient for sulfatides in brain and kidney and for indoxyl-3-sulfate and tyrosine-O-sulfate in urine. We did not find any striking differences.

We are studying the problem of whether normal urine contains any S-sulfo-cysteine. In addition, an extensive screening is in progress to evaluate 3,000 patients in an institution for the mentally retarded to detect additional cases of sulfite oxidase deficiency.

2. Pancreatic insufficiency and hyperparathyroidism (Drs. Andrew L. Warshaw and Leonard Laster, NIAMD)

We mentioned last year our patient with malabsorption due to pancreatic insufficiency who, after treatment with pancreatic extract, was found to have a parathyroid adenoma. Removal of the adenoma appears to have been associated with an improvement in her ability to absorb fat. This patient was reported in the literature during the past year.

3. The catabolism of gastrin tetrapeptide in vitro (Drs. John H. H. Walsh, NCDC, and Leonard Laster, NIAMD)

A major accomplishment of recent years was the isolation of gastrin, the elucidation of its structure, and the synthesis of the molecule by Gregory and his associates. Gastrin is a single chain polypeptide made up of 17 amino acids. A pyroglutamyl residue at its N terminus makes it resistant to aminopeptidase, and an amide group on the C-terminal phenylalanine makes it resistant to carboxypeptidase. The C-terminal tetrapeptide, Try-Met-Asp-Phe-amide, possesses the full range of physiologic actions of the entire molecule, although in reduced potency. When the amide is missing from the C-terminal tetrapeptide, the molecule is physiologically inert. We have been exploring the inactivation of this polypeptide hormone fragment by tissue extracts, and have discovered an amidase that will catalyze removal of the C-terminal amide from BOC-Try-Met-Asp-Phe-amide. A quantitative method for measuring this enzymatic activity was developed, and appropriate conditions for the assay were determined. The pH optimum was found to be around 6.7. The enzyme is active in liver and intestinal mucosa, less active in spleen and pancreas, and absent from diaphragm. Additional studies of the deamidase activity are in progress. It may represent an important mechanism for the inactivation of gastrin *in vivo*.

4. Developmental aspects of enzymes involved in transsulfuration (Drs. Joseph Volpe, NICHD and Leonard Laster, NIAMD)

A study is in progress to determine the pattern of development of cystathionine synthase in liver and brain of the rat, and to analyze the development and regional distributions of the enzyme and of cystathionine in primate brain. The influence of various hormones on the developmental pattern of the rat enzyme is also under study.

Significance to Bio-medical Research and the Program of NIAMD:

These studies have resulted in the detection of previously unrecognized diseases, in the elucidation of their underlying biochemical defects, and in the demonstration of pathways for the metabolism of physiologically important compounds.

Proposed Course:

The general outline of these studies will be continued.

Honors and Awards:

The studies on gastrin tetrapeptide catabolism were presented by invitation at a Symposium on Gastrin and Related Synthetic Peptides, sponsored by the American Society for Pharmacology and Experimental Therapeutics at the meetings of the Federation of American Societies for Experimental Biology in Atlantic City in April 1968. The work was also accepted for presentation at the Annual Meeting of the American Gastroenterological Association in Philadelphia, May 1968.

Publications:

Irreverre, F., Mudd, S. H., Heizer, W. D., and Laster, L.: Sulfite oxidase deficiency: Studies of a patient with mental retardation, dislocated ocular lenses, and abnormal urinary excretion of S-sulfo-L-cysteine, sulfite, and thiosulfate. Biochem. Med. 1: 187-217, Nov. 1967.

Warshaw, A. L., Heizer, W. D., and Laster, L.: Pancreatic insufficiency as the presenting feature of hyperparathyroidism. Ann. Intern. Med. 68: 161-167, Jan. 1968.

Clinical Endocrinology Branch

Work in the Branch has continued in several major areas: thyroid biochemistry, polypeptide hormones, mechanisms of hormone action and protein structure. New studies on insulin biochemistry are related to the Branch's expanding interest in diabetes.

One senior member, Dr. Glenn Mortimore, left in September 1967 to become Associate Professor of Physiology at the new Pennsylvania State College of Medicine in Hershey, Pennsylvania. Visiting workers from abroad during 1967-8 included three from Italy and one from Japan. Three others (from Belgium, Japan and Italy) left at the end of the summer of 1967.

I. Thyroid Biochemistry

A. Iodide Transport

The basic protein isolated from cobra venom, cobramine B, has been further characterized by amino acid analysis (52 residues, mol wt 5840). Its action to inhibit thyroid iodide transport has been shown to be due mainly to increased iodide efflux. Membrane transport of other substances also is affected. The action seems to be due to interaction with negative surface charges on the thyroid cell and is also produced by other polycations such as protamine and polylysine. Polyanions prevent these effects, but do not readily reverse them once they have developed. (Wolff, Larsen, H. Salabe).

B. Thyroxine Synthesis and Iodination Reactions

Further characterization of the oxidized intermediate in the synthesis of thyroxine from hydroxydiiodophenylpyruvic acid and diiodotyrosine was provided by nuclear magnetic resonance. The enol form of the pyruvic acid derivative, and not the keto form, was the one most efficiently converted to thyroxine. A similar intermediate has been found in the synthesis of 3,5,3'-triiodothyronine from hydroxymonoiodophenylpyruvic acid and diiodotyrosine. The yield of triiodothyronine was about 8%, considerably below that for thyroxine. It is still unknown whether this mechanism or the direct coupling of iodotyrosine residues is involved in thyroid hormone biosynthesis. (Cahnmann, Funakoshi, Nishinaga).

Iodination of tyrosyl residues in vitro in native bovine thyroglobulin has been compared with that in succinylated bovine thyroglobulin (composed of 17S and 12S species) and of 12S guinea pig thyroglobulin produced by dissociation. The residues in succinylated thyroglobulin behaves more like free tyrosine in that monoiodotyrosine formation is greater and passes through a maximum. This study provides further evidence on the role of protein structure in the formation of biologically important iodoamino acids. (Edelhoch, Nissley).

C. Iodoproteins

Further efforts have been made to define the subunit structure of thyroglobulin. The monomer chains in reduced alkylated bovine thyroglobulin have been separated by centrifugation into two components. These did not differ in amino acid content, iodoamino acid composition or electrophoretic mobility. Other chemical comparisons are now in progress. A more thorough knowledge of thyroglobulin chemistry is required for progress to be made in studying its biosynthesis and physiological role in hormone formation. (Edelhoch, Nissley).

Progress in the purification of the components of the abnormal thyroglobulin found in transplantable rat thyroid tumor has been hampered by the failure thus far to obtain stable preparation in good yield. The crude fractions, however, have been examined for iodoamino acid content, and were found to contain mono- and diiodotyrosine but not thyroxine or triiodothyronine. A survey of human thyroid nodules has thus far revealed no instance of abnormal thyroglobulin resembling that found in bovine congenital goiter and in the rat tumor. (Robbins).

D. Protein Synthesis in the Thyroid Gland

After perfecting methods for the preparation of polyribosomes from bovine thyroid, their nature was examined by density gradient centrifugation and by electron microscopy. Although the best current evidence indicates an unusually large monomer molecular weight (165,000) for thyroglobulin, the polyribosome pattern from thyroid did not show an unusual quantity of large polyribosomes compared to other tissues. The region near the bottom of the density gradient tubes showed a rather uniform distribution of polyribosomes containing 20 to 30 units, which is about one-half that expected from earlier evidence for a monomer of 165,000 molecular weight. Either current theories on polyribosome size do not apply to thyroid, or the subunit of thyroglobulin is smaller than indicated by present evidence.

Labelled amino acid incorporation into protein *in vitro* was only slightly more effective in larger polyribosome fractions. A portion of the newly synthesized protein proved to be immunochemically related to thyroglobulin although, for various reasons, it did not have the physical properties of the native molecule. This protein, however, was recognized by antibodies which did not react with native thyroglobulin but rather with antigenic sites available only on denatured forms of thyroglobulin. These immunological findings have an important bearing on studies of *in vitro* protein synthesis in general (Robbins, Rall, Kondo, De Nayer, G. Salabe).

E. Measurement of Iodocompounds in Biological Fluids

Adaptation of iodimetry methods to iodoamino acid analysis in plasma continues to present technical problems. Anion exchange resins used to separate iodoamino acids have been found to be badly contaminated with iodine, and the chloric acid used in digestion has been found to produce an inhibitor which profoundly affects the cerate-arsenite reaction. Nevertheless, a micro method permitting measurement of subnanogram quantities of iodine has been perfected, which can be performed with as little as 12 µl of serum. (Lewallen).

Application of gas liquid chromatography to iodoamino acid analysis has been developed, employing trimethylsilyl derivatives. The method has proved useful in studies of thyroxine synthesis reactions since it is more effective than other chromatographic methods in separating various intermediates. Using an electron capture detector, as little as 200 picograms of iodoamino acid can be detected, and this should lead to a method useful for biological fluids. (Cahnmann, Funakoshi).

F. Thyroid Hormone Action

Because of the many thyroid hormone effects in mitochondria, an investigation of their effects on mitochondrial RNA has been undertaken. Preliminary to this, RNA synthesis in isolated rat liver mitochondria has been investigated. Labelled ribonucleotide triphosphates are incorporated into low molecular weight (4-5S) RNA with a high preponderance of U neighbors, but containing the four common bases. A mitochondrial RNA polymerase has been isolated and appears to make a similar product (Wolff, Rall, Varrone).

G. Studies in Thyroid Disease

Because of the difficulties encountered in supervising thyroid hormone replacement therapy in the Marshall Islands population which had developed thyroid desease after fallout radiation, a study was initiated to evaluate the use of weekly doses of thyroxine. A group of patients requiring thyroid hormone replacement (patients with total thyroidectomy for thyroid carcinoma, with hemithyroidectomy for thyroid nodules, or with lymphocytic thyroiditis) were given the weekly requirement of thyroxine (2.1 mg) in a single dose per week or in the usual divided dose given once each day. No untoward reaction to this single large dose each week was encountered. By the criteria of blood hormone level and clinical response, the patients were euthyroid and as well maintained on the single weekly dose as on the usual daily dose and thyroid uptake of radioiodine was suppressed. The weekly dosage schedule was, therefore, initiated by the Brookhaven team under Robert Conard on their latest survey mission to the Marshall Islands. (Robbins, Bernstein).

II. Polypeptide Hormones

A. Nature of the Hormones

It is becoming increasingly likely that for each of the polypeptide hormones, additional forms of the hormone, other than the one classically ascribed to it, may exist during synthesis, storage, release, or in the circulation. Characterization of the heretofore unknown entities should clarify our understanding of physiology and of diseases in man. Studies in man and in the dog, on the nature of insulin in blood have shown that insulin is released from the pancreas in two forms. One component is identical with the classical form of pancreatic insulin. The second component has a larger molecular size, is immunologically related to pancreatic insulin but not identical, and is released later following stimulation of insulin release. Future studies on insulin physiology must take into account this heterogeneity in circulating insulin but the biological role of the various components is at present unknown (Roth, Gorden, Pastan).

With the development of a rapid specific immunoassay for vasopressin (Roth, Klein, Peterson) studies have focused on possible alternate forms of that hormone in the pituitary and the hypothalamus, in tumors that produce vasopressin, and in the circulation. Such alternate forms are in the process of being characterized. (Robertson, Roth, Gorden).

Gonadotropins have been studied in an analogous fashion. A sensitive radioimmunoassay for gonadotropins has been developed (Rosen, Schlaff, Roth). By use of this assay, a new distinctive form of gonadotropin-like substance has been detected in urine, blood and tumor of a patient with a metastatic carcinoid (Rosen, Engelman). Patients with malignant lung tumors are being studied extensively to characterize their capacity to produce gonadotropins. Interestingly in one patient, hormone production was by far the earliest sign to indicate that the patient harbored a malignancy. Wider surveys are underway to see if hormone tests will be useful in early detection of cancer. (Rosen, Becker).

Other tumors studied extensively included (1) an ACTH-producing carcinoid in a patient who also had acromegaly due to a pituitary tumor (2) a gonadotropin-producing choriocarcinoma in a patient whose plasma also showed thyroid stimulating activity.

B. Mode of Action

Earlier studies (Pastan, Roth, Macchia) have shown that the first step in the action of polypeptide hormones is an equilibrium between hormone in solution and attached to a fixed number of sites on the cell surface. Other steps that occur shortly thereafter are activation of the enzyme, adenyl cyclase, and accumulation of cyclic 3',5'-AMP. The latter substance then appears to act as the intracellular mediator of the hormone's action. Several investigators are actively characterizing these early steps, with particular attention being paid to detect steps preceding adenyl cyclase activation.

Present studies have extended observations of ACTH effect on the adrenal slices to subcellular fractions. ACTH activation of adenyl cyclase has been demonstrated in whole homogenates as well as in highly purified membrane fractions. In addition to subcellular localization of the hormone-sensitive enzyme, the optimal conditions for its assay have been worked out. By the use of specific ACTH-antibody, recent experiments have strongly suggested the existence of at least one step between binding of hormone and adenyl cyclase activation. (Taunton, Roth, Pastan).

Analogous results have been observed with TSH. TSH, in cell free preparations of the thyroid, activates adenyl cyclase (Pastan). Propranolol and phentolamine, adrenergic blocking agents, block the effect of TSH on adenyl cyclase. Dibutyryl cyclic AMP, an effective analogue of cyclic AMP, still acts in the thyroid in the presence of the adrenergic blocking agents, strongly suggesting that these blocking agents act on steps very early in TSH action (Levey, Roth, Pastan).

Earlier studies have shown that certain bacterial products presumably by action at the cell can mimic the action of TSH both in vivo and in vitro. (Pastan, Macchia). Two of these components from c. perfringens have been purified and are in the process of being characterized. One that is only active in vitro is a sphingomyelinase. The other, active in vivo, is under further study. These enzymes should shed a great deal of light on the process by which hormones can activate cells (Pastan, Macchia, Katzen).

Phospholipase C, derived from Clostridia, makes thyroid cells insensitive to TSH (Pastan and Macchia). These observations have been extended to other systems, especially that of viruses and infected cells. Thus chick embryo fibroblasts, treated with this enzyme, do not show replication of Semliki Forest virus but otherwise function normally, including interferon formation. In addition to providing information on cell surface phenomena and the role of phospholipids, in vivo applications to combating viral infections may be derived. (Pastan, Friedman).

Similarly studies of the role of cyclic AMP as an intracellular mediator of hormone action have been broadened to E. coli. In these organisms cyclic AMP specifically stimulates the synthesis of three inducible enzymes (β-galactosidase, tryptophanase and D-serine deaminase) and prevents their repression following exposure to glucose. In addition to its importance in understanding induction phenomena, this finding gives us a more general appreciation of cyclic AMP controlled processes. (Pastan, Perlman).

C. Diseases of the Anterior Pituitary Gland

A prospective study of 17 patients with acromegaly, irradiated with 4000r to the pituitary gland, has been completed. In contrast to untreated patients, the irradiated patients demonstrated a fall in plasma growth hormone levels after one to two years. This correlated with improved glucose tolerance and insulin sensitivity, and a fall in serum phosphorous. Estrogen therapy in large doses also improved glucose tolerance and insulin sensitivity but did not affect plasma growth hormone concentration. (Roth, Gorden).

III. Protein Structure

In order to understand at a more fundamental level the important effects of protein structure on its optical properties, a detailed study of low molecular weight models containing tyrosine and tryptophane has been pursued. Diketopiperazine derivatives containing either tyrosyls, tryptophanyls, or both, in *cis* or *trans* configuration, have been studied. Fluorescence yield was greatly increased in dimethylsulfoxide or dioxane compared to water. Circular dichroism revealed that the magnitude of the ellipticities in these compounds is several times greater than in linear dipeptides containing these chromophores. The consequent improved resolution of dichroic bands should prove very useful in the assignment of similar bands in proteins.

Fluorescence polarization of a series of glycyl-tryptophanyl peptides were measured in order to evaluate the effect of chain size on relaxation time of the indole side chain. Similar data were obtained with biologically important polypeptides containing 24 residues (an active ACTH analog), 40 residues (glucogon) and 90 residues (parathyroid hormone). The results indicate that these polypeptide chains have little or no structure (Edelhoch, Nissley, Bernstein, Wilchek).

Serial No. NIAMD/CEB 1c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Nonenzymic Synthesis of Thyroxine (T_4) and 3,5,3'-Triiodothyronine (T_3)

Previous Serial Number: CEB-3c, 2c, 1c

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: A. Nishinaga, Ph.D., H. Kon, Ph.D., K. Funakoshi, Ph.D.

Cooperating Units: PB-NIAMD

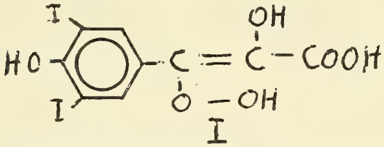
Man Years

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

The reactions of 3,5-diiodotyrosine (DIT) with its keto acid analog (DIHPPA) and with that of MIT (MIHPPA) are nonenzymic models for the biosynthesis of T_4 and T_3. Both reactions require oxygen. We reported in last year's research report that kinetic studies led to the conclusion that in the reaction with DIHPPA, the keto acid reacts first with oxygen to form a T_4-precursor which then reacts anaerobically with DIT to form T_4.

Subsequently optimal conditions for the conversion of DIHPPA to the T_4-precursor as well as the latter's chemical behavior were further investigated. A study of the keto-enol tautomerism of DIHPPA, mainly by means of nmr, showed that only the enol form is efficiently converted to the T_4-precursor. The pH optimum for the conversion is 7.2 - 7.6. The previously proposed structure I for the T_4-precursor was confirmed by further studies on the chemical behavior. Thus I is not only reduced to the corresponding glycol by sodium borohydride, but also converted anaerobically to 4-hydroxy-3,5,-diiodobenzaldehyde and oxalic acid, while DIHPPA is stable to strong alkali in the absence of oxygen, but rapidly converted to the same two reaction products with the uptake of 1 mole of oxygen in the presence of the latter.


I

MIHPPA was converted to a T_3-precursor by the uptake of 1 mole of oxygen. In this case the pH optimum is higher (∿8.5) because the pK of the phenolic group is higher for MIHPPA than for DIHPPA (only the phenolate ion is capable of taking up oxygen). Reaction of the T_3-precursor with DIT gave T_3 in ∿8% yield.

It is assumed that the phenoxyl radicals of MIHPPA and of DIHPPA are intermediates in the conversion of these keto acids to the T_3 or T_4-precursor. The phenoxyl radical of DIHPPA was studied by means of esr-spectroscopy. It was found that it can exist, like the keto acids themselves in a keto and enol form. Both are, as expected, very short-lived.

Honors and Awards: None

Publications:

1. Nishinaga, A., Kon, H., Cahnmann, H. J.: Model reactions for the biosynthesis of thyroxine. XI. The nature of a free radical formed in the autoxidation of 4-hydroxy-3,5-diiodophenylpyruvic acid. J. Org. Chem. 33: 154-162, Jan. 1968.

2. Nishinaga, A., Cahnmann, H. J., Kon, H., and Matsuura, T.: Model reactions for the biosynthesis of thyroxine. XII. The nature of a thyroxine precursor formed in the synthesis of thyroxine from diiodotyrosine and its keto acid analog. J. Org. Chem. 7: 389-397, Jan. 1968.

Serial No. NIAMD/CEB 2c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Gaschromatographic Analysis of Iodinated Compounds

Previous Serial Number: None

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: K. Funakoshi, Ph.D.

Cooperating Units: None

Man Years

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

A large number of iodinated compounds were determined qualitatively and quantitatively by means of gas liquid chromatography. These compounds comprise not only the known iodoamino acids and their desamino analogs, but also position and side chain analogs and intermediates in the synthesis of T_3 and T_4. A few compounds could be analyzed directly, without derivatization, all other compounds were converted to their trimethylsilyl derivatives. Treatment of the iodinated compounds with O,N-bis(trimethylsilyl)acetamide results in the trimethylsilylation of all functional groups in a single reaction which represents an advantage over other methods of derivatization. Using a hydrogen flame ionization detector, a few micrograms are usually analyzed while with an electron capture detector as little as 200 picograms can be determined in most cases. Preliminary experiments for the determination of T_3 and T_4 in term have been carried out.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 3c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Characterization of a Growth Factor Required by Trypanosomes, a Study in Species Specificity.

Previous Serial Number: None

Principal Investigator: H. J. Cahnmann, Ph.D., C. L. Greenblatt, M.D.

Other Investigators: None

Cooperating Units: LPD-NIAID

Man Years

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Trypanosoma lewisi grows in rats, but not in mice. When, however, rat serum is injected into mice, the latter become suitable hosts. It seemed therefore likely that rat serum contains a species-specific growth factor. The present study was undertaken to gain an insight into the nature of that factor. Rat serum was fractionated by Na_2SO_4 precipitation. The precipitate was chromatographed on DEAE cellulose. The active fraction, was further chromatographed on Sephadex G-200, and the most active fraction obtained in this fractionation was chromatographed on DEAE Sephadex. An active fraction was obtained which promotes fatal trypanosome infections in the mouse and is homogeneous in the analytical ultracentrifuge ($S_{20,w}=7$) as well as in disc. gel and paper electrophoresis. Immunoelectrophoresis, however, revealed two distinct precipitin lines, which require further identification. Treatment of a crude preparation from rat serum with cyanogen bromide did not destroy the activity which means that it may be possible to fragment the above mentioned highly active protein fraction without loss of activity.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 4c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Physical Chemistry of Proteins

Previous Serial Number: CEB 4c, 3c, 2c

Principal Investigator: H. Edelhoch, Ph. D.

Other Investigators: R. Lippoldt, P. Nissley, M.D., M. Wilchek, Ph. D., R. Bernstein, M. D.

Cooperating Units: LCB-NIAMD

Man Years

Total: 3 3/4
Professional: 3 1/4
Other: 1/2

Project Description:

The question of whether or not the iodination pattern of thyroglobulin subunits is different from that of native thyroglobulin was studied by iodination of succinylated calf (17S and 12S species) and 12S guinea pig thyroglobulin. The latter was produced by dissociation of the native species. There was a major difference in the MIT curve for succinylated calf thyroglobulin compared to native due either to structural or size differences produced by succinylation. In the case of guinea pig 12S species, the iodination pattern was identical to that of guinea pig 19S thyroglobulin.

Characteriation of reduced alkylated thyroglobulin monomer chains, isolated by sucrose gradient centrifugation, has continued utilizing the following approaches: 1) amino acid analysis; 2) disc gel electrophoresis; 3) measurement of tyrosine, MIT, DIT, and T_4 by spectral titration; 4) Ouchterlony gel diffusion. Isolated monomer chains have not been proven to be chemically different by these techniques and studies are currently in progress to compare the monomer chains by end group determination and by the cyanogen bromide method.

The circular dichroism of a) mono-and di tyrosyl diketopiperazine in neutral and alkaline pH; b) mono-and di tryptophanyl diketopiperazine; and two mixed diketopiperazines has been measured in water, DMSO and dioxane. The magnitude of the ellipticities is several times that found with the linear dipeptide forms of these molecules. As a consequence better resolution of dichroic bands have been obtained for the phenol and indole chromophores than hitherto published. The spectra should prove to be very useful in assignment of C.D. bands in proteins.

The quantum yields of a) mono- and dityrosyl (cis and trans forms) diketopiperazines; b) mono-and ditryptophanyl (cis and trans forms) diketopiperazines; and c) two mixed diketopiperazines have been measured in water, DMSO and dioxane. Major increases in yields were found in DMSO and dioxane.

The fluorescence polarization of a series of glycyl-tryptophanyl peptides were measured in order to evaluate the effect of chain size on relaxation time of the indole side chain. Similar data were obtained on polypeptide chains of 24 residues (an active ACTH analog), 40 residues (glucagon) and 90 residues (parathyroid hormone) in order to determine if these molecules were globular or linear. These molecules appear to be polypeptide chains with little or no structure.

Honors and Awards: None

Publications:

1. Edelhoch, H.: Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry 6: 1948-1954, Feb. 1967.
2. Perlman, R. L., and Edelhoch, H.: The formation of diiodotyrosine in iodinated human serum albumin. J. Biol. Chem. 242: 2416-2422, Jan. 1967.
3. van Zyl, A., and Edelhoch, H.: The function of the protein in the control of DIT synthesis. J. Biol. Chem. 242: 2423-2427, Jan. 1967.
4. de Crombrugghe, B., Edelhoch, H., Beckers, C., and De Visscher, M.: Thyroglobulin from human goiters. J. Biol. Chem. 242: 5681-5685, July 1967.
5. Salvatore, G., Aloj, S., Salvatore, M., and Edelhoch, H.: Hybridization of half molecules of guinea pig thyroglobulin. J. Biol. Chem. 242: 5002-5007, Nov. 1967.
6. Perlman, R. L., van Zyl, A., and Edelhoch, H.: The properties of thyroglobulin XVI energy transfer to iodoamino acids. J. Amer. Chem. Soc. 90: 2168-2172, Apr. 1968.
7. Edelhoch, H., and Perlman, R. L.: The properties of thyroglobulin XVII. The differential reactivity of the MIT and DIT residues in thyroglobulin with N-acetyl midazole. J. Biol. Chem. 243: 2008-2011, Apr. 1968.

Serial No. NIAMD/CEB 5c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Measurements of Iodocompounds in Biological Materials.

Previous Serial Number: CEB-5c, 6c

Principal Investigator: C. G. Lewallen, M.D.

Other Investigators: K. Vogel, M. Gentry, M. Brackbill, and Hans Hoch, Ph.D.

Cooperating Units: Veterans Administration Center, Martinsburg, West Va.

Man Years

Total: 2 1/4
Professional: 2
Other: 1/4

Project Description:

Objectives and Methods are as previously described in report for 1965-1966.

Major findings this year. 1) Analytical grade anion exchange resins commercially available in the acetate cycle have been found to be badly contaminated with iodine. Values obtained by wet ashing of resin range from 3.9-8.4 nanograms per milligram of dry resin.

2) A serious problem encountered in anion exchange chromatography of iodocompounds is contamination of the effluent by material originating in the resin itself capable of giving a positive cerate-arsenite response. This material has been identified as iodide by paper chromatography.

3) In the chloric acid (wet ashing) procedure for iodine microanalysis, the temperature at which digestion is carried out and the volume of chloric acid used profoundly alter the quantitative aspects of the catalytic effect of iodide on the cerate-arsenite reaction. This is due to the formation of an inhibitor during wet ashing. In collaboration with Dr. Hans Hoch a paper is in preparation describing the formation and properties of this inhibitor.

4) In collaboration with Dr. Hans Hoch it has been possible to scale down the chloric acid, cerate-arsenite method for iodine to permit

quantitation of subnanogram quantities. PBI measurements on 12 μl serum are routinely performed. A manuscript covering this work has been completed but not yet presented for publication: Hoch, H., and Lewallen, C.G.; Microdetermination of iodine in serum.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 6c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of Action of Thyroid Stimulating Hormone

Previous Serial Number: 8c, 9c

Principal Investigator: Ira Pastan, M.D.

Other Investigators: G. Levey, M.D., V. Macchia, M.D., R. Katzen

Cooperating Units: None

Man Years

Total: 2 1/2
Professional: 2 1/4
Other: 1/4

Project Description:

Objectives: To define the initial site of interaction of TSH with thyroid tissue and characterize the early steps in TSH action.

Major Findings: In cell free preparations of thyroid tissue, TSH increases the production of cyclic 3',5'-AMP by increasing the activity of the enzyme adenyl cyclase. This activation is specific for TSH and can be detected thirty seconds after TSH addition. Propranolol and phentolamine block this action of TSH and also block the action of TSH on glucose oxidation in the thyroid. This is consistent with the hypothesis that increased levels of cyclic 3',5'-AMP are responsible for increased glucose oxidation.

Previously, we found that Clostridium perfringens produces a material that stimulates thyroid activity. This material has been further purified and resolved into two components. One has been identified as a spingomyelinase which increases glucose oxidation and phospholipid turnover in thyroid slices but is not active *in vivo*. The other substance which is active *in vivo* is being characterized further.

Proposed Course: Since we previously found that TSH binds to a superficial site on the thyroid cell one would predict that adenyl cyclase and the mechanism whereby TSH increases its activity are under investigation.

Serial No. NIAMD/CEB 6c

Honors and Awards: None

Publications:

1. Pastan, I., and Wollman, S. H.: Colloid droplet formation in dog thyroid in vitro. J. Cell Biol. 35: 262-266, 1967.

2. Pastan, I., and Katzen, R.: Activation of adenyl cyclase in thyroid-stimulating hormone. Biochem. Biophys. Res. Commun. 29: 792-797, Dec. 1967.

3. Pastan, I., and Macchia, V.: Mechanism of thyroid-stimulating hormone action. J. Biol. Chem. 24: 5757-5761, Dec. 1967.

4. Macchia, V., Bates, R., and Pastan, I.: The purification and properties of a thyroid-stimulating factor isolated from Clostridium perfringens. J. Biol. Chem. 16: 3726-3730, Aug. 1967.

Serial No. NIAMD/CEB 7c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of Cyclic 3',5'-AMP in E. coli.

Previous Serial Number: None

Principal Investigator: I. Pastan, M. D.

Other Investigators: R. Perlman, M. D., R. Katzen

Cooperating Units: None

Man Years

Total: 2
Professional: 1 1/4
Other: 3/4

Project Description:

Objectives: To determine the role of cyclic 3',5'-AMP in the control of the synthesis of inducible enzymes in E. coli.

Major Findings: Cyclic 3',5'-AMP stimulated the synthesis of β-galactosidase when induced with isopropylthiogalactoside, tryptophanase when induced by tryptophan and D-serine deaminase when induced by D-serine. Cyclic 3',5'-AMP also prevents the repression of these enzymes by glucose. The action of cyclic 3',5'-AMP is highly specific since it fails to stimulate overall protein or RNA synthesis or the synthesis of two derepressible enzymes (tryptophan synthetase and alkaline phosphatase).

Proposed Course: To define the mechanism by which cyclic 3',5'-AMP regulates the synthesis of these enzymes.

Honors and Awards: None

Publications:

1. Perlman, R., and Pastan, I.: Cyclic 3',5'-AMP: Stimulation of β-galactosidase and tryptophanase induction in E. coli. Biochem. Biophys. Res. Commun. 30: 656-663, Mar. 1968.

Serial No. NIAMD/CEB 8c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of Phospholipids in Viral Replication

Previous Serial Number: None

Principal Investigator: I. Pastan, M.D.

Other Investigators: R. M. Friedman, M.D.

Cooperating Units: LP-NCI

Man Years

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Objectives: To evaluate the role of phospholipids in viral replication.

Major Findings: Phospholipase C has been highly purified. When chick embryo fibroblasts are treated with the enzyme the replication of Semliki Forest Virus is prevented. The enzyme alters cellular membranes so that virus replication, which occurs on membranes, is greatly depressed. However, cellular functions such as RNA and protein synthesis and the production of interferon are not affected by enzyme treatment.

Proposed Course: To extend the studies with phospholipase C to other viruses and to evaluate its use as an antiviral agent *in vivo*.

Honors and Awards: None

Publications:

1. Pastan, I., and Friedman, R. M.: Actinomycin D: Inhibition of phospholipid synthesis in chick embryo cells. Science 160: 316-317, April 1968.

2. Friedman, R. M., and Pastan, I.: Specific inhibition of viral growth in cells treated with phospholipase C. Proc. Natl. Acad. Sci., Apr. 1968.

Serial No. NIAMD/CEB 9c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Thyroid Iodoproteins

Previous Serial Number: CEB-10c, 9c, 10c

Principal Investigators: J. Robbins, M. D.

Other Investigators: P. DeNayer, M.D., and B. Weathers

Cooperating Units: None

Man Years

Total: 2 1/4
Professional: 2
Other: 1/4

Project Description:

Progress on the purification of the components of the abnormal thyroglobulin found in transplantable rat thyroid tumor (Wollman, line 1-8) has been hampered by the failure thus far to obtain stable preparations in good yield. The protein, detected by labelling with radioiodine, is apparently present in small amount and becomes insoluble when higher degrees of purity are reached. The crude fractions have, however, been examined for iodoaminoacid content by radiochromatography of pronase hydrolysates. The abnormal thyroglobulin contains monoiodotyrosine and diiodotyrosine, but no thyroxine of triiodothyronine could be detected.

A method of thin-layer gel filtration for the study of iodoproteins was developed, using the transplantable tumors as sources of different types of iodoprotein. It was possible to separate thyroglobulin from smaller iodoproteins such as iodoalbumin and to identify them by their position in autoradiographs. The abnormal thyroglobulin was excluded from the gel, as was thyroglobulin, despite a slow sedimentation rate resembling that of albumin.

A survey of human functioning thyroid nodules of varying types obtained at surgery has now included 8 patients. Thus far, no instance of abnormal thyroglobulin resembling that found in bovine congenital goiter and in rat thyroid tumor has been recognized in man.

Serial No. NIAMD/CEB 9c

Honors and Awards: None

Publications:

1. DeNayer, P., Weathers, B., and Robbins, J.: Thin-layer gel filtration of thyroid iodoproteins. Studies on rat transplantable thyroid tumors. Endocrinology 81: 1118-1124, Nov. 1967.

Serial No. NIAMD/CEB 10c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Protein Synthesis in the Thyroid Gland

Previous Serial Number: CEB-4c, 5c

Principal Investigators: Y. Kondo, D.S.c., J. E. Rall, M.D., Ph.D., and J. Robbins, M. D.

Other Investigators: P. DeNayer, M.D., and G. Salabe, M. D.

Cooperating Units: DIR-NIAMD

Man Years

Total: 2
Professional: 2
Other: 0

Project Description:

After perfecting methods for the preparation of polyribosomes from the very fibrous bovine thyroid gland, the nature of these structures was examined by density gradient centrifugation and by electron microscopy. This study is of special interest since thyroid is a tissue which produces a large amount of a single protein species, thyroglobulin, which, according to the best available evidence, has an unusually large monomeric unit of 165,000 molecular weight. Contrary to expectations, it was not possible to obtain preparations containing polyribosomes predominantly of large size. Most polyribosomes patterns resembled those seen in other tissue such as liver, and some patterns contained predominantly hexameric units. The region near the bottom of the density gradient tubes, however, were shown by electron microscopy to contain a rather uniform distribution of polyribosomal structures containing 20 to 30 units. This is about one-half the size expected from studies by others on the polyribosomes involved in synthesis of other proteins, such as hemoglobin, γ-globulin and myosin. This may indicate that current theories are not applicable to thyroid or that the thyroglobulin subunit is smaller than indicated by current evidence.

Labelled amino acids were incorporated in vitro into protein by all sizes of polyribosomes, and a portion of this protein was shown to be related immunochemically to thyroglobulin. Thyroglobulin synthesis was somewhat more effective in the larger polyribosomes, but did not occur exclusively in the large-size fractions. As expected, the native thyroglobulin molecule could not be demonstrated since the synthetic system required the presence of β-mercaptoethanol which, by reduction of disulfide bonds, might be expected to hinder their formation. Furthermore, much of the protein was strongly adherent to polyribosomes and could be solubilized only by agents which denature thyroglobulin. The polyribosome system also is not expected to carry out carbohydrate chain synthesis, and mature thyroglobulin contains about 10% carbohydrate. An additional unexpected problem was the observation that glutamic acid was converted to glutamine by the protein synthesis system. Neither glutamic acid nor glutamine was incorporated into protein.

The precipitation of newly synthesized thyroglobulin-related protein by anti-thyroglobulin antibodies was shown not to be inhibited by native thyroglobulin in excess. This finding led to a study of the immunochemical reaction which revealed that antiserum against highly purified native thyroglobulin contains a mixture of antibodies, some of which react with antigenic sites on the native molecule, and some with sites which are reactive only in denatured thyroglobulin (detergent-treated, partially hydrolized, or reduced and alkylated). These altered forms of thyroglobulin were capable of inhibiting the precipitation of polyribosomal-synthesized protein by anti-thyroglobulin serum, confirming the thyroglobulin nature of the newly synthesized protein.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 11c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Thyroxine - Protein Interactions

Previous Serial Number: 10c

Principal Investigators: J. Robbins, M.D.

Other Investigators: R. Bernstein, M.D., R. Berger, M.D., J. E. Rall, M.D.,Ph.D.

Cooperating Units: DIR-NIAMD, LTD-NHI

Man Years

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

Further attempts have been made to measure the kinetics of the interaction of thyroxine with serum albumin using an improved stop-flow apparatus developed by Robert Berger of NHI. The quenching of albumin fluorescence by bound thyroxine is used as the index of binding. Despite considerable effort, the binding kinetics could not be measured, and this work will continue when further improvement of the apparatus is achieved. The ultimate aim is to measure the interaction kinetics with thyroxine-binding globulin and prealbumin.

Studies have been initiated on the genetic polymorphism of thyroxine-binding prealbumin in Macacus rhesus monkeys. This polymorphism had been detected some years ago in collaborative studies with Baruch Blumberg. We recently have obtained sera from breeding monkeys and their offspring, and have found the three forms of prealbumin, detected by electrophoretic mobility differences, in this colony. Work is in progress to determine the mode of inheritence of this polymorphism.

Honors and Awards: None

Publications:

1. Condliffe, P. G., and Robbins, J.: Pituitary thyroid stimulating hormone and other thyroid stimulating substances. In Gray, C. H. and Bacharach, A. L. (Eds.): Hormones in Blood. 2 ed. New York, Academic Press, 1967, vol. 1, pp. 334-376.

2. Robbins, J., and Rall, J. E.: The iodine-containing hormones. In Gray, C. H. and Bacharach, A. L. (Eds): Hormones in Blood. 2 ed. New York, Academic Press, 1967, vol. 1, pp. 383-470.

Serial No. NIAMD/CEB 12c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Thyroid Disease

Previous Serial Number: None

Principal Investigators: J. Robbins, M.D.

Other Investigators: P. Gorden, M.D., R. Bernstein, M.D., C. Becker, M.D.

Cooperating Units: None

Man Years

Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Because of the difficulties encountered in supervising thyroid hormone replacement therapy in the Marshall Islands population which had developed thyroid disease after fallout radiation, a study was initiated to evaluate the use of weekly doses of thyroxine. A group of patients requiring thyroid hormone replacement (patients with total thyroidectomy for thyroid carcinoma, with hemithyroidectomy for thyroid nodules, or with lymphocytic thyroiditis) were given the weekly requirement of thyroxine (2.1 mg) in a single dose per week or in the usual divided dose given once each day. No untoward reaction to this single large dose each week was encountered. By the criteria of blood hormone level and clinical response, the patients were euthyroid and as well maintained on the single weekly dose as on the usual daily dose and thyroid uptake of radioiodine was suppressed. The weekly dosage schedule was, therefore, initiated by the Brookhaven team under Robert Conard on their latest survey mission to the Marshall Islands.

Unusual drug reactions in patients under study for thyroid disease were encountered. One was the development of drug-induced hepatitis resulting from tapazol (mercaptoimidozol) in a patient with Graves' disease while receiving massive doses of adrenal steroid for malignant exophthalmos. The other was the development of an extreme degree of thyroid swelling and pain, resembling severe acute thyroiditis, in a patient given bovine TSH in a standard test of thyroid function. These were considered of sufficient importance to be made the subjects of case reports.

Honors and Awards: None

Publications:

1. Conard, R. A., Meyer, L. M., Sutow, W. W., Robertson, J. S., Rall, J. E., Robbins, J., Jesseph, J. E., Deisher, J. B., Hicking, A., Lawi, I., Gusmano, E. A., and Eicher, M.: Medical survey of the people of Rongelap and Utirik Islands eleven and twelve years after exposure to fallout radiation (March 1965 and March 1966). Washington, D. C., 1967, 164 p.

2. Robbins, J., Rall, J. E., and Conard, R. A.: Late effects of radioactive iodine in fallout. Ann. Int. Med. 66: 1214-1242, June 1967.

Serial No. NIAMD/CEB 13c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Gonadotropin Studies

Previous Serial Number: CEB-11c

Principal Investigator: Saul W. Rosen, Ph.D., M.D.

Other Investigators: C. Becker, M.D., Ida Stotler, R. Winand, M.D.

Cooperating Units: LBP-NIAMD

Man Years

Total: 3 1/4
Professional: 2
Other: 1/4

Project Description:

A) Bronchogenic Carcinoma With Gynecomastia

Blood and urine from 53 patients with known bronchogenic carcinoma has been submitted to us by various investigators throughout the United States. In 4 cases bioassay of the urine and radioimmunoassay of the serum showed abnormally large amounts of gonadotropic activity. Although these cases were not randomly selected, the substantial percentage of presumed gonadotropin producers in this pilot study mandates a controlled prospective analysis of the incidence of gonadotropin (and other polypeptide hormone) production in carcinoma of the bronchus. We plan to initiate such a project in two large hospitals and hope to screen concurrently patients with other kinds of cancer to assess the reality of the reported singular capability of bronchogenic carcinoma for ectopic hormone synthesis.

Two additional patients with bronchogenic carcinoma and gynecomastia but no increased concentration of gonadotropin in blood, urine or tumor have been studied. Etiology of their gynecomastia is unexplained.

B) Ectopic Gonadotropin Production by Other Tumors

Urine, blood and tumor from a young woman with metastatic carcinoid of the stomach has been studied through the courtesy of Dr. Karl Engelman.

Although the urinary excretion of bioassayable gonadotropin was within normal limits, her serum contained very high concentrations (>16,000 ng equivalent of FSH/ml; normal <10) of a material which displaced FSH-I^{131} from rabbit anti-FSH in our double antibody radioimmunoassay. The patient's serum did not cause release of radioactivity from FSH-I^{131} and did not interfere with the reaction between rabbit gamma globulin (RGG) and sheep anti-RGG. Yet this "specific" interaction with anti-FSH at the FSH-binding site was qualitatively and quantitatively unlike those we have found with TSH, LH, FSH and HCG for which curves of displacement of FSH-I^{131} as a function of hormone concentration are superimposable. Furthermore, the biologic activity of this serum (mouse uterus, rat ovarian weight augmentation) was negligible compared to sera from patients with comparable (or even less) immunoassayable gonadotropin. We are studying the possibility that this patient's tumor was making a new kind of gonadotropin.

C) Long acting Thyroid Stimulator(LATS) and Human Chorionic Gonadotropin(HCG)

We have found very high concentrations of LATS (8-10 x control at 2 hours, 15-17 x control at 17 hours) in serum from a patient with (probable) primary extragenital choriocarcinoma. This man excreted massive amounts of HCG or HCG-like material in the urine (10-40 million IU daily) and had very high serum concentration (10,000 IU/ml) of this HCG. Roughly comparable amounts (∿2500 IU) of a crude (SA 2670IU/mg) commercial preparation of HCG also showed potent (6x control at 2 hours, 9x control at 7 hours) LATS activity. Detailed studies are in progress.

Honors and Awards: None

Publications:

1. Rosen, S. W., and Gahres, E.: Nonpuerperal galactorrhea and the contraceptive pill. Obstet. Gynec. 29: 730, 1967.

Serial No. NIAMD/CEB 14c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Nature of Plasma Insulin in Man

Previous Serial Number: None

Principal Investigator: J. Roth, M.D.

Other Investigators: P. Gorden, M.D., M. Lesniak, C. Hendricks

Cooperating Units: None

Man Years

Total: 2 1/2
Professional: 3/4
Other: 1 3/4

Project Description:

Endogenous circulating insulin in man has two components, both of which are secreted by the pancreas. One component ("little insulin") is not distinguished from purified porcine (pancreatic) insulin by gel filtration or by its reaction with a guinea pig anti-porcine insulin serum. The second component ("big insulin") is less retarded on gels and reacts somewhat differently with this anti-insulin serum; its molecular weight is estimated to be 8,000-9,000. Shortly after glucose is fed, most of the insulin in plasma is "little insulin" whereas 1-2 hours after glucose, up to 50% of plasma insulin in this immunoassay is "big insulin." A prolonged fast prior to glucose administration (starvation diabetes) appears to enhance the output of "big insulin." It is as yet unclear whether "proinsulin", the presumed intra-pancreatic precursor of insulin, is related to the "big insulin" in plasma.

Honors and Awards: None

Publications:

1. Roth, J., Gorden, P., and Pastan, I.: "Big insulin": a new component of plasma insulin detected by immunoassay. Proc. Nat. Acad. Sci. July 1968. (in press).
2. Roth, J., Gorden, P., and Pastan, I.: "Big insulin": a new component of plasma insulin in man. J. Clin. Invest. (in press) (Abstract)
3. Gorden, P., and Roth, J.: A new component of plasma insulin detected by radioimmunoassay. Program of the 28th Meeting of the American Diabetes Assoc. (Abstract)

Serial No. NIAMD/CEB 15c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunoassay of Vasopressin

Previous Serial Number: 4c

Principal Investigator: G. Robertson, M.D., J. Roth, M.D.

Other Investigators: P. Gorden, M.D., I. Pastan, M.D.

Cooperating Units: None

Man Years

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

A sensitive and specific radio-immunoassay for arginine vasopressin (AVP) has recently been developed in this laboratory, (J. Clin. Invest. 45: 1064, 1966) and its application to the measurement of plasma hormone levels is currently under investigation. It has been used successfully to measure AVP in the plasma and in tumors of patients with the syndrome of inappropriate ADH secretion.

This immunoassay technique has also been used to search for other naturally occurring polypeptides that are immunologically related to AVP. We have been able to detect two such components from hypothalamus, both of which have molecular weights significantly greater than the native hormone. Work is now under way to purify and further characterize these substances.

Honors and Awards: None

Publications:

1. Klein, L. A., and Roth, J.: Antidiuretic hormone metabolism studied by radio-immunoassay. Surgical Forum 18: 289-291, 1967.

2. Marks, L., Berde, B., Klein, L. A., Roth, J., Goonan, S. R., and Nabsheth, D. C.: Inappropriate vasopressin secretion and carcinoma of the pancreas. Amer. J. Med. (in press).

Serial No. NIAMD/CEB 16c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interaction of ACTH with Adrenal Cells

Previous Serial Number: CEB-15c

Principal Investigator: O. David Taunton, M.D., J. Roth, M.D.

Other Investigator: E. King

Cooperating Units: None

Man Years

Total: 2 1/2
Professional: 1 1/4
Other: 1 1/4

Project Description:

Having established that the first step in ACTH action is an equilibration between ACTH in the medium and specific sites on the external surface of the adrenal cell, we have continued our studies on the mechanism of action of ACTH. We have recently found that ACTH stimulates adenyl cyclase activity in homogenates of normal rat adrenals and functional adrenal tumors. Since adenyl cyclase, catalyses the formation of cyclic 3',5'-adenosine monophosphate (cyclic AMP) this is additional evidence in favor of the proposal that cyclic AMP is an intracellular mediator of ACTH action.

We have studied the effect of ACTH on adenyl cyclase in functional adrenal tumor in detail and have found that the optimal conditions for assaying the enzyme activity include pH 7.7, Mg^{++}:ATP of 1.0-1.5, and ATP concentration of 2.5 mM. Our findings are consistent with suggestions by others that the enzyme is localized to the plasma membrane.

Preliminary work with anti-ACTH antibody indicates that the activation of adenyl cyclase is preceded by one or more steps after binding of ACTH, since the ACTH effect on adenyl cyclase can be partially reversed with antibody after brief exposure to ACTH. On prolonging exposure to ACTH up to 60 minutes, antibody is without effect. These studies with antibody are being pursued.

Serial No. NIAMD/CEB 16c

Honors and Awards: None

Publications:

1. Taunton, D., Roth, J., and Pastan, I.: ACTH stimulation of adenyl cyclase in adrenal homogenates. Biochem. Biophys. Res. Commun. 29: 1-7, Oct. 1967.

2. Taunton, O. D., Roth, J., and Pastan, I.: The first step in ACTH action: Binding to tissue. J. Clin. Invest. 46: 1122, 1967 (abstract)

3. Taunton, O. D., Roth, J., and Pastan, I.: ACTH activation of adenyl cyclase in purified adrenal membranes. J. Clin. Invest. (in press) (abstract).

2

Serial No. NIAMD/CEB 17c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of Human Growth Hormone (HGH) in Man

Previous Serial Number: 3c

Principal Investigators: P. Gorden, M.D., J. Roth, M.D.

Other Investigators: C. Hendricks, M. Lesniak

Cooperating Units: None

Man Years

Total: 1
Professial: 1/2
Other: 1/2

Project Description:

1. A thorough prospective study was completed in which seventeen patients with acromegaly were irradiated with 4000r. Plasma HGH measured by immunoassay fell by 1-2 years late. Similar effects were noted in acromegalics who also had adrenal or thyroid diseases. Untreated acromegalic subjects showed no change or a rise in plasma HGH.

2. Correlations were observed in individual patients with acromegaly between plasma HGH and (a) glucose tolerance, (b) insulin sensitivity, and (c) serum phosphorous both before and after pituitary irradiation.

3. Estrogens in large doses were administered orally to acromegalics. Plasma HGH did not change but glucose tolerance and insulin sensitivity improved markedly.

Honors and Awards: None

Publications:

1. Roth, J., Bates, R. W., and Gorden, P.: Immunoassay of plasma growth hormone in acromegaly. International Symposium on Growth Hormone, Sept. 1967. International Congress No. 142. Excerpta Medical Foundation. 1967, pp. 6-7. (abstract)

2. Roth, J., Gorden, P., and Bates, R. W.: Studies of growth hormone and prolactin in acromegaly. Proc. Inernatl. Symp. on Growth Hormone Milan, 1967. (in press).

Serial No. NIAMD/CEB 18c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Thyroidal Iodide Transport

Project Serial Number: CEB-17c, 16c

Principal Investigator: J. Wolff, M.D., Ph.D.

Other Investigators: M. Salabe, M.D., M. M. Ambrose, P. R. Larsen, M.D., E. Gormley

Cooperating Units: None

Man Years

Total: 2 3/4
Professional: 1 1/2
Other: 1 1/4

Project Description:

Object--to define membrane components involved in iodide transport.

Cobra venoms contain heat stable, basic, proteins that inhibit iodide accumulation by thyroid slices. Activity is not related to the content of phospholipase A or various other enzymatic activities. These proteins are present only in Elapid venoms and not in snakes of the Crotalidae or Viperidae families.

Two such basic proteins, named cobramines A and B, have been purified from the crude venom of the Indian cobra (Naja naja) by carboxymethyl cellulose chromatography, gel filtration, and ammonium sulfate crystallization. Together they constitute ~40% of the venom protein. Their isoelectric points are ~11.9 and ~12.6, respectively.

Cobramine B was found to be homogeneous in paper and disc gel electrophoresis and by ultracentrifugation ($S_{20,w} = 0.98$). The molecular weight was 6400 from equilibrium centrifugation measurements and 5840 on the basis of amino acid analysis (52 residues).

Purified cobramine B from Naja naja inhibits the accumulation of I^- by thyroid and parotid slices or isolated choroid plexus. Accumulation of TcO_4^- and ReO_4^- is also inhibited. The effect has a rapid onset, is temperature-dependent, and is prevented by polyanions or antivenom, but it is reversed with great difficulty, presumably because of very firm binding to tissue. Pretreatment with neuraminidase does not influence the inhibition.

The major portion of the inhibition is due to increased I^- efflux from the slices. Significant increases occur at 2μg per ml, and I^- efflux is a more sensitive measure of the cobramine B effect than is I^- accumulation. Cobramine B leads to K^+ loss from the cell and increases the rate of equilibration of the sucrose or inulin spaces.

Inhibition of I^- accumulation is also produced by protamine, histones, polyarginine, polyornithine, and polylysine, but not by lysozyme or ribonuclease A. Tetralysine is ineffective, and a polylysine of average molecular weight 14,000 or greater is maximally effective. Larger polymers are not more potent. As with cobramine B, inhibition is prevented but not easily reversed by polyanions.

It is postulated that cobramine B interacts with the negative surface charges of the thyroid cell.

CMC chromatography has also permitted separation of 5 distinct toxic proteins which together account for ~90% of the toxic activity of cobra venom. Three of these are more potent than the crude venom. Phospholipase A does not contribute to the lethal effect in mice.

Honors and Awards: None

Publications:

1. Larsen, P. R., and Wolff, J.: Inhibition of accumulation transport by a protein from cobra venom. Biochem. Pharmacol. 16: 2003-2009, 1967.

2. Larsen, P. R., and Wolff, J.: The basic proteins of cobra venom I. Isolation and characterization of cobramines A and B. J. Biol. Chem. 243: 1283-1289, Mar. 1968.

3. Wolff, J., Salabe, H., Ambrose, M., and Larsen, P. R.: The basic protein of cobra venom II. Mechanism of action of cobramine B on thyroid tissue. J. Biol. Chem. 243: 1290-1296, Mar. 1968.

Serial No. NIAMD/CEB 19c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Role of Thyroid Hormones in Mitochondrial Synthesis

Previous Serial Number: None

Principal Investigators: J. Wolff, M.D., Ph.D., S. Varrone,M.D., J. E. Rall,M.D.

Other Investigator: None

Cooperating Units: DIR-NIAMD

Man Years

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

Because of the many thyroid hormone effects in mitochondria, an investigation of the effects of these hormones on mitochondrial RNA was undertaken. The optimal conditions for nucleotide incorporation into isolated rat liver mitochondrial under sterile conditions were determined as a precondition for the study of hormonal effects. Such mitochondria incorporate labelled ribonucleotide triphosphates into a RNA (low molecular weight 4S, and 5S) fraction which contains the 4 common bases. Nearest neighbor analysis shows a high preponderance of U neighbors. The reason for this has not yet been fully elucidated. A mitochondrial polymerase has been isolated. It appears to make a similar product.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 20c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanisms of Thyroxine Action

Previous Serial Number: None

Principal Investigators: J. Wolff, M.D., Ph.D., R. L. Perlman, M.D.,Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

During studies on the effect of thyroxine on dehydrogenases it was found that dimethyl sulfoxide (DMSO) inhibits horse liver alcohol dehydrogenase. In the direction of aldehyde reduction, this inhibition is competitive with aldehyde, with a K_i of 5×10^{-3} M. DMSO reacts with the enzyme-NADH binary complex to form a highly fluorescent ternary complex, with a dissociation constant similar to the K_i. The inhibition of aldehyde reduction can be interpreted as being due to competition between aldehyde and DMSO for the carbonyl binding site of the enzyme-NADH binary complex.

Honors and Awards: None

Publications:

1. Perlman, R. L., and Wolff, J.: Dimethyl sulfoxide: An inhibitor of liver alcohol dehydrogenase. Science 160: 317-318, Apr. 1968.

Most of biochemical, immunologic and physiologic investigations conducted by this Branch the past year have been directly or indirectly related to cystic fibrosis of the pancreas. This generalized disease due to an unknown inborn error of metabolism indeed has been the object of much interest and intensive studies both in this Institute and elsewhere and significant advances towards a better understanding of its elusive pathogenesis have been made in the past year.

Investigations also have been pursued in other types of recently recognized and rare pancreatic disorders in the pediatric age group, which need much further clarification from the standpoint of etiology and pathogenesis as well as from the descriptive clinical standpoint.

A. BIOCHEMICAL, IMMUNOLOGIC AND PHYSIOLOGIC STUDIES OF SUBMAXILLARY GLANDS AND SALIVA

The submaxillary gland is involved in patients with CF. Morphologic changes in the gland are present at autopsy, submaxillary saliva from patients with CF is abnormal in appearance and analytic studies have revealed an elevated content of many inorganic, organic and enzymatic components. Submaxillary saliva offers the opportunity of obtaining for biochemical and immunologic investigations an uncontaminated glycoprotein-rich secretion in patients with CF without admixture of other bacterial or excretory products. It also affords the possibility of investigating the physiologic function of another exocrine gland in CF in addition to the sweat gland, which is the only other for which data are available so far.

All of the submaxillary studies have been performed in collaboration with the Dental Services Branch of the Dental Research Institute. In all instances submaxillary saliva was collected by a modified Schneyer technique with segregates of methyl-methacrylate constructed individually. A number of patients with CF ranging in age from 12 to 30 years were studied, but in addition, a number of normal volunteers of the same age group were investigated as there was almost no information in the literature as to normal values.

1. The Role of Calcium in Submaxillary Saliva of Patients with Cystic Fibrosis

Chemical studies have confirmed previous observations by another group of investigators that submaxillary saliva in patients with systic fibrosis is turbid compared to the clear secretions obtained from normal control subjects, and that levels of calcium as well as total protein, hexoses, fucose, uronic acid and hexosamine are increased, while the levels of sodium, chloride and potassium are not significantly different from those of normal subjects.

In order to get a better insight into the possible interaction between the increased calcium and some of the organic constituents, studies by means of acrylamide-gel electrophoresis were conducted. It was found that by decreasing calcium levels some bands which appeared weak or absent in cystic saliva as compared to normal saliva reappeared. Conversely the electrophoretic appearance of normal saliva could be transformed into a pattern quite similar to that of cystic fibrosis by increasing calcium concentration to levels comparable to those in patients with cystic fibrosis. Studies with specific antisera showed no immunologic differences between the various bands while no immunologic differences were found between fractions from cystic or normal saliva. Chemical analysis of eluted bands showed glycoproteins to be major constituents.

It was concluded that submaxillary saliva in both normal subjects and patients with cystic fibrosis contains as major constituents glycoproteins, probably identical, which combine or polymerize with calcium present in (>1,000,000) that cannot travel in the gel.

This represents the first time an attempt has been made to study the effect of abnormal ionic concentrations in CF on the solubility of glycoproteins in this disease. Since in cystic fibrosis widespread obstruction or organ passages gives rise to most of the pathologic and clinical findings the reaction just outlined may be one of the main mechanisms involved. This hypothesis is supported by the finding of high calcium levels as well as an increased total protein content in many glycoprotein-rich biologic fluids from patients with this disorder.
Drs. Gugler, Pallavicini, di Sant' Agnese, NIAMD: Dr. Swerdlow, NIDR

2. Immunological Studies of Submaxillary Saliva from Patients with CF and Normal Controls

Little was known not only in patients with CF but even in normal subjects about the presence and levels in submaxillary saliva of serum proteins and their fractions, of immunoglobulins and in particular of IgA, which is the main immunoglobulin of the respiratory tract and of exocrine glands. In addition, it was still uncertain as to how many antigenic components from the salivary gland tissue pass into the saliva in both normal subjects and patients with CF, and lastly whether or not in this site it is possible to detect an antigenic component specific for CF.

In a search for an answer to these many questions submaxillary saliva as well as tissue extracts of submaxillary glands from patients with CF and normal controls were analyzed and compared by immunodiffusion methods. Specific anti-sera to submaxillary saliva and submaxillary gland tissue in both CF patients and controls were prepared in rabbits and donkeys. A number of specific antisera against various serum and other protein fractions also were obtained from other investigators in addition to some that were commercially available.

It was demonstrated that in submaxillary saliva the following plasma proteins were present: prealbumin, albumin, alpha-antitrypsin, transferrin, and the three immunoglobulins, IgA, IgG, IgM; albumin and IgA being the main components. Orosomucoid, alpha$_1$-glycoprotein, ceruloplasmin, alpha$_2$-macroglobulin and fibrinogen could not be readily identified.

All protein fractions were present in similar amounts in submaxillary saliva from normal individuals and patients with CF. The only exception was IgA which was significantly elevated in submaxillary saliva as well as in the serum of patients with CF.

Ten specific components were identified in submaxillary gland tissue. One of them was shown to be alpha-amylase. Of the nine other tissue antigens found in submaxillary gland extracts, four were considered to be gland specific. The submaxillary saliva antigen as well as the tissue antigens were demonstrated to be immunologically identical in the two groups and no antigen specific for CF was found.

In this investigation much information was obtained as to the immunologic composition of submaxillary saliva in both normal subjects and patients with CF. It was found for the first time that IgA is increased in the secretions of CF patients, an increase which is analogous to that found in parotid saliva and in the serum of these patients and which seems to reflect the degree of pulmonary involvement. This speaks in favor of passage of this immunoglobulin from the serum to the salivary gland and to submaxillary saliva, rather than an in situ production. The results of the present studies also confirm the findings of the previously extensive and systematic immunologic investigations, most of them conducted in this laboratory, that failed to uncover an antigenic determinant specific for CF in various body fluids and tissue extracts.
Drs. Gugler, Pallavicini, di Sant' Agnese, NIAMD; Drs. Swerdlow, Zipkin, NIDR.

3. Studies of the Physiology of the Submaxillary Gland

Adequate amounts of submaxillary saliva can be collected for a variety of organic and inorganic components to be analyzed in the same sample. In serial sampling over a long period of time, it is possible to study changes due to the varying rate of secretion as well as to exhaustion or fatigue of the gland.

Nine patients with CF and nine normal controls were used in these studies. Atomic absorption spectrophotometry, colorimetric and enzymatic methods, polyacrylamide gel and continuous flow electrophoresis and immunodiffusion methods were used. A very large number of determinations were performed thus far.

A large variation in submaxillary maximal water output was observed in both patients and normals. However, in contrast to the normals a progressive reduction in water output over a period of several months was observed in some patients with CF. In these studies it was shown that not only sodium and chloride are flow rate dependent, but also potassium, magnesium, and calcium, although they respond in a different manner. Although sodium concentration rose in linear fashion with increasing flow rate, two different patterns of sodium secretion were observed in normal subjects. One group consistently secreted this ion in concentrations roughly double that of the other group. In patients with CF the response was similar, but three groups of secretory patterns could be distinguished. As a whole sodium concentrations were not significantly different in the two groups.

Conversely, potassium, and magnesium concentrations were inversely related to water output and no significant difference was found between the response of the two groups compared.

In both normal subjects and patients with CF inorganic phosphorus decreased with increasing flow rate. However, in some patients with CF this fall is much delayed, so that with increasing flow rate the values remained higher in cystics than in normals.

In normal subjects calcium concentration is high at very low flow rates and decreases at first when the rate increases, but at flow rates higher than 0.5 ml per minute the values increase again. This behavior of calcium in relation to flow rate is probably related to the behavior of proteins in relation to rate in submaxillary saliva. Some patients with CF had a calcium which was much higher than normal subjects and rose at times up to 15 mEq/L. However, in other patients with CF calcium concentrations were within normal limits over the full range of flow rates.

In normal subjects and in patients with CF total protein concentration and especially the so-called specific salivary proteins increase with increasing rates in similar fashion. Total hexoses, fucose and sialic acid concentration also increased with flow rate but much less dramatically than in the case of the proteins. Conversely, albumin and IgA, which were shown in previous studies to be the two major fractions of serum proteins present in saliva, decreased with increasing water output to reach a constant plateau at a flow rate of approximately 1 ml per minute. Uric acid also behaved very much like the serum proteins.

These physiologic studies add considerably to our knowledge of cystic fibrosis. Heretofore only the sweat gland had been studied from the standpoint of physiology, but not a mucous-producing exocrine gland. These investigations for the first time give an insight in the function of such a gland in this disorder; as in the case of the sweat gland the results in many instances were surprisingly normal. It appears as if some patients with CF are normal from the submaxillary standpoint regardless of involvement of other areas of the body. With the passage of time the submaxillary gland often appears to be involved in the process characteristic of this disease; the concentrations of protein and calcium then seem to rise as the flow rate decreases. It is at this point that calcium and proteins probably interact to form a precipitate which blocks the ducts and initiates a rapid worsening of the disease process. The submaxillary gland is presumably only a model of what happens in other areas in cystic fibrosis and causes pancreatic achylia, chronic pulmonary disease, hepatic cirrhosis and other obstructive complications.
Drs. Wiesmann, Pallavicini, di Sant' Agnese, Handwerger, Peter, NIAMD:
Dr. Swerdlow, NIDR.

4. Studies of "Mucus" in Normal Human Submaxillary Saliva

Reflex stimulated saliva was pressue filtered at room temperature through a 0.05μ average pore-size membrane. The effluent was non-viscous, usually clear and qualitatively contained most, if not all of the salivary proteins. On the membrane a white rubber-like layer was deposited. This material was dissolved in twice the original volume of water and centrifuged to remove a small amount of debris from the viscous filament-forming supernatant - Fraction 1. (Fraction 1 can be separated from saliva also by centrifugation at 38000 x g for 10 minutes either at 4° or 35°C. Evidence that its presence in saliva is not dependent upon the temperature condition of its isolation.) The supernatant, made to 40% - 50% ethanol was centrifuged to remove traces of remaining insoluble material and the supernatant made to 66% ethanol. After standing overnight at 4°C a precipitate was centrifuged down and dissolved with water to the original volume - Fraction 2. This fraction, though still viscous, had lost its filament-forming character.

Fraction 1 in immunodiffusion analysis gave precipitation line similar to those produced by total saliva, wheras Fraction 2 gave only one line. Chemical analysis of Fraction 2 showed 13.8% galactose, 19.8% fucose, 1.5% sialic acid, 7.9% hexosamine and 57.0% protein. Following acrylamide-gel electrophoresis of Fraction 2 at pH 8.4 a strong PAS reaction was observed only in the sample gel, while two distinct protein lines were visible in the running gel. If, prior to electrophoresis, Fraction 2 was treated with EDTA a pronounced additional protein band appeared in the running gel. This result suggests that some protein is associated in some manner with the Schiff-positive glycoprotein.

It was concluded that in submaxillary saliva there is a complex system of proteins, glycoproteins and electrolytes. The glycoproteins are able to aggregate to very large units, which can be filtered and centrifuged and can bind proteins, presumably by calcium bridges, to form even larger complexes. Although the nature of this link is not fully understood the proteins appear to protect these large glycoprotein molecules against chemically-induced precipitation and denaturation. Drs. Pallavicini, Wiesmann, NIAMD: Dr. Swerdlow, NIDR.

B. OTHER BIOCHEMICAL AND IMMUNOLOGICAL STUDIES

1. Investigations of Ekkrinosiderophilin

The iron in human skim milk is not free but is chelated to a specific protein, ekkrinosiderophilin, which, as shown by Heremans and others, is the iron-binding protein common to many glandular secretions. With the milk ekkrinosiderophilin (lactosiderophilin, lactotransferrin, lactoferrin) may be found varying but limited quantities of plasma siderophilin, among other plasma proteins. Lack of agreement in the literature as to some physicochemical properties of ekkrinosiderophilin required investigation into those characteristics of both the isolated protein and of that as present in skim milk. The specific absorption curve of iron ekkrinosiderophilin, either as purified complex or in skim milk, has its absorption maximum at approximately $\lambda 465 m\mu$ rather than $\lambda 450 m\mu$. Electrophoretic mobilities of the protein constituents of skim milk, of the milk "albumin fraction" of Szuchet-Derechin and Johnson, and of the isolated ekkrinosiderophilin on cellulose acetate in barbital buffer, pH 8.5, or in polyacrylamide gel at pH 8.4 warrant this iron-binding protein to be considered electrophoretically as a gamma rather than beta globulin. The molecular weight of purified iron-ekkrinosiderophilin as determined by sedimentation and diffusion analysis assuming a partial specific volume of 0.73 is 82,500 - 83,000. Iron analysis of 100% iron saturated ekkrinosiderophilin of known weight show that 2 atoms of iron are chelated by each molecule of the protein. The sedimentation constant S of purified iron ekkrinosiderophilin at 0.5% concentration in 0.05-M phosphate buffer at pH 7.5 and 25°C was determined by optical ultracentrifugal analysis to be 5.33. The $S^{0725}_{20.W}$ value of the iron-binding complex in skim milk as analyzed by the sucrose gradient method of Martin and Ames is about 8.9. The higher sedimentation constant of the iron-binding component in skim milk compared to that of the isolated ekkrinosiderophilin, some evidence of change in electrophoretic behavior of the isolated protein when mixed with skim milk, and varying precipitability of DEAE-cellulose fractionated ekkrinosiderophilin by dialysis against distilled water suggest that this iron-binding protein may exist in milk in association with some other protein constituent.

The main objective of this study was to obtain a highly purified preparation of ekkrinosiderophilin for further studies of the antimicrobial action of this protein, important in the defense of the body against infection. This study is of special interest in cystic fibrosis as this protein is present in all exocrine glands. As a result of the present chemical, physico-chemical and immunologic investigations some of the controversies in the literature were resolved and the way opened for further study of this compound.
Drs. Schade, NIAID: Drs. Pallavicini, Wiesmann, NIAMD.

2. Tissue Antigens and Enzymes Found in Normal Human Urine

Donkey antisera prepared against human liver, pancreas, submaxillary gland homogenates and rabbit anti-human urine, were tested against extracts of human liver, pancreas, heart, submaxillary gland, skeletal muscle, lung kidney and urine. All antibodies to plasma proteins were removed by thorough absorption, so that only tissue antigens were detected. Immunodiffusion analyses were carried out with antigen extracts adjusted to equal protein concentrations.

Numerous tissue antigens were found in human urine concentrations with these antisera. For example, anti-pancreas serum revealed seven reactions with urine, and only three of these were detected with kidney extract. On the other hand, this serum showed that urine shared at least four tissue antigens with submaxillary gland. Anti-liver serum showed fewer reactions with urine, and these appeared to be more widely distributed in other tissues.

It was noted that several of these antigens present in the urine appeared to be in higher concentrations than in the extracts of the tissue which served as the immunizing antigen.

Preliminary tests of the enzyme activities of the immune precipitates revealed an esterase in urine with the anti-pancreas sera which was not apparent in any of the pancreas-anti-pancreas bands. Reciprocal analysis with anti-urine serum demonstrated one esterase which was shared with all tissues tested. Glucuronidase activity was shown to be present in urine and liver but not in kidney, in the immune precipitates formed by extracts of these tissues with the anti-submaxillary gland antiserum.

It is planned to extend these studies to materials on patients with CF in order to complete the immunologic studies in this disorder. These investigations also will provide the necessary basis for further study of a material of normal antigenic activity which appears to be present in larger amounts in patients with CF than in normal controls in both urine macromolecular and tissue homogenate studies.
Drs. Halbert, Greene, University of Miami: Dr. Pallavicini, NIAMD.

C. CLINICAL STUDIES

1. Unusual Insulin Secretion and Glucose Intolerance Characteristic of Cystic Fibrosis

Glycosuria and abnormal glucose tolerance test have been seen with increasing frequency as the life span of patients with CF is becoming more prolonged and a number of adolescents and young adults with this disease have been observed. In the past, this intolerance has been attributed to classical diabetes mellitus, although there were many reasons to doubt this conclusion.

Cystic fibrosis, with or without impaired glucose tolerance, was characterized by insulinopenia following oral glucose, but adequate insulin release following other stimuli. In 31 patients, 7 to 28 years old, oral glucose tolerance was normal or slightly impaired in 64%, moderately impaired in 26%, and severely impaired in 10%. Following oral glucose, insulin secretory patterns (immunoassay) revealed (1) patients with normal or slightly impaired tolerance had a prompt but subnormal rise in insulin, (2) patients with moderately impaired tolerance had a delayed as well as subnormal response, and (3) patients with severe intolerance had a negligible rise. When glucagon or tolbutamide was infused at the height of the insulin response to oral glucose, serum insulin promptly increased 100% or more in all cases, irrespective of blood glucose changes. Other studies showed (1) much less impaired glucose and insulin responses to intravenous glucose as compared to oral glucose, (2) normal insulin tolerance, (3) normal growth hormone responses to hypoglycemia, and (4) no effect of phentolamine on insulin secretion.

The glucose intolerance is not classical diabetes; family history, characteristic complications and post-mortem signs of diabetes were absent. In contrast to the cystics, young patients with diabetes mellitus and hypoinsulinemia failed to secrete further insulin when glucagon, tolbutamide, or arginine was infused at the height of the insulin response to oral glucose. We conclude that the glucose intolerance in cystic fibrosis is due to a defect in the mechanism whereby oral glucose stimulates insulin release.

Pathologic examination of the islets of Langerhans in 35 patients, including the seven patients in this study, was performed. Islet tissue was only moderately decreased, but fibrosis was noted in most islets. This fibrosis separated beta cells from each other, from adjacent alpha cells, and from neighboring blood vessels. We feel that this fibrosis, by altering the synergistic effect of the agents known to augment insulin secretion, could possiblily explain the unusual insulin secretion as well as the glucose intolerance in the patients with CF studied by us.

This represents the first comprehensive investigation of this important complication of CF using the techniques for radio-immuno-assay of serum insulin levels as well as pathologic studies. It was demonstrated that the glycosuria which has been seen increasingly with the advancing age of CF patients in recent years is not due to classical diabetes mellitus, but is due to disruption of the islet architecture because of the increasing fibrosis of the pancreas. These conclusions have an important bearing on the pathogenesis and treatment of cystic fibrosis. They may also influence medical thinking in the evaluation of so-called "diabetes mellitus" in other conditions characterized by pancreatic fibrosis such as chronic recurrent pancreatitis.
Drs. Handwerger, Roth, Gorden, di Sant' Agnese, Peter, NIAMD: Dr. Carpenter, NCI.

2. Albumin Metabolism in Cystic Fibrosis

Hypoalbuminemia in CF occurs most commonly in moderately or severely ill patients with advanced pulmonary disease. In spite of the frequency of this finding, its cause has not been elucidated. In view of the frequency of malabsorption, hepatic involvement, and cor pulmonale in cystic fibrosis, hypoalbuminemia in this disease could result from hyposynthesis due to either malabsorption of amino-acids or to liver disease; hypocatabolism of proteins secondary to protein-losing enteropathy; and/or dilution of a normal albumin pool due to increase plasma volume secondary to the chronic pulmonary disease and cor pulmonale.

To investigate the etiology of hypoalbuminemia in cystic fibrosis ^{125}I and ^{51}Cr albumin metabolic studies were performed on 19 patients between the ages of 8 and 25 years and 5 normal controls. Twelve patients were hypoalbuminemic (serum albumin<3.6 mg%) and seven patients had normal concentrations of serum albumin (>3.6 mg%). The mean values for total circulating albumin, albumin synthetic rate, fractional catabolic rate, and intravascular-extravascular albumin distribution in hypoalbuminemic patients were not significantly different from the mean values of the normoalbuminemic patients or the controls. However, the plasma volume of the 10 hypoalbuminemic patients (57.4±9.4 cc/kg) was significantly greater than that of the 6 normoalbuminemic patients (43.6±7.0 cc/kg) or of the controls (42.0±3.2 cc/kg). Two of 6 patients with cystic fibrosis and hepatic disease had mildly decreased albumin synthetic rates in addition to an increased plasma volume. Seven of 10 hypoalbuminemic patients and none of the normoalbuminemic patients had slightly increased gastrointestinal loss of ^{51}Cr albumin, but this protein loss was only a minor factor in the pathogenesis of hypoalbuminemia.

The major cause of hypoalbuminemia in cystic fibrosis patients beyond infancy is, therefore, the dilution of a normal albumin mass in an enlarged plasma volume. Clinical and post-mortem studies established that this hypervolemia probably is secondary to cor pulmonale. In addition, decreased albumin synthesis can on occasion result from extensive hepatic involvement in cystic fibrosis

The present study represents the first comprehensive and careful investigation of albumin metabolism and hypoalbuminemia in cystic fibrosis. It is apparent from the results reported that a low serum albumin in patients with CF and severe pulmonary disease is usually due to hemodilution because of increased blood volume secondary to cor pulmonale. Although directly related to the severity of the pulmonary disease, hypoalbuminemia may appear days or months before it is clinically apparent that cardiac involvement is present and as such may be a simple and invaluable guide to prognosis and treatment in this disease.
Drs. Peter, Handwerger, di Sant'Agnese, NIAMD: Drs. Strober, Waldmann, NCI.

3. Studies in Pancreatic Disease Other Than Cystic Fibrosis

Clinical, biochemical and pathologic studies are being carried out on two siblings with pancreatic insufficiency and bone marrow dysfunction. This is a newly described syndrome in children and is characterized by dwarfism, pancreatic insufficiency, a normal sweat test, no chronic pulmonary involvement and a variety of hematological abnormalities, most commonly thrombocytopenia and cyclical neutropenia. Only a few cases of this interesting and rare disease entity have been reported so far, but detailed biochemical and especially pathologic studies are lacking. In addition our patients had many features (notably hepatic cirrhosis and portal hypertension) never reported heretofore in this syndrome.

In collaboration with the Section on Gastro-enterology, NIAMD, intensive clinical and biochemical studies are being conducted on a large family with chronic inherited pancreatitis. This will represent the seventh kindred with this recently recognized disease to be described.
Drs. di Sant'Agnese, Laster, Handwerger, Loeb, Peter, NIAMD.

Serial No. NIAMD:PMB:1c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunologic and Biochemical Studies in Cystic Fibrosis

Previous Serial Number: None

Principal Investigator: Paul A. di Sant' Agnese, M.D.

Other Investigators: Edouard Gugler, (guest worker); Charles Pallavicini, Ph.D.; Stuart Handwerger, M.D.; Georges Peter, M.D.; Irene Schulz, (guest worker); Herbert Swerdlow and Isadore Zipkin, NIDR.

Cooperating Units: Dr. Seymour P. Halbert, University of Miami; Dr. Robert H. Parrott, Children's Hospital, Washington, D.C.; Dr. Samuel S. Spicer, Medical School of South Carolina, Charleston, South Carolina; Drs. Jack Orloff, Mauriceburgh, Laboratory of Kidney and Electrolytes, NHI; Drs. Robert Gordon, Sidney Wolfe, Metabolic Diseases Branch, NIAMD

Man Years:

Total:	1 3/4
Professional:	3/4
Other:	1

Project Description:

Objectives:

Three different projects were undertaken on sweat and submaxillary glands and the Tamm-Horsfall urinary glycoprotein in an attempt to elucidate the pathogenesis of cystic fibrosis:

1. The sweat gland defect in CF is well recognized and gives rise to an increase in sodium, chloride and potassium content of sweat. Conversely, most of the other solutes and many physiologic parameters of the sweat gland function are normal. In order to answer some of the questions related to the composition of the precursor fluid these studies of the sweat gland were undertaken.

2. The submaxillary gland is known to be involved in cystic fibrosis. Submaxillary saliva in fibrocystics has been shown to be turbid compared with a clear secretion in normal subjects and to have an increase in many organic components. Submaxillary saliva is a glycoprotein-rich secretion which offers the opportunity for many biochemical and immunologic studies.

3. The Tamm-Horsfall urinary glycoprotein is easily separable by methods that do not affect its chemical or immunologic properties and is a relatively simple fraction - a logical model system to test the hypothesis of a structural abnormality of glycoproteins in cystic fibrosis.

Methods Employed:

A variety of chemical methods (e.g.: continuous flow and acrylamide-gel electrophoresis, colorimetric determinations, ect.) are of immunodiffusion techniques were used in these investigations. In addition, micropuncture techniques of human sweat glands under disecting scope microscopy were used. The Komnick pyroantimonate method for ultrastructural localization of sites presumably high in sodium was carried out on skin biopsies of patients with CF and normal subjects before and after thermal sweating.

Major Findings:

The Role of Calcium in Submaxillary Saliva: In order to get a better insight into the possible interaction between the increased calcium and some of the organic constituents, studies by means of acrylamide-gel electrophoresis were conducted. It was found that by decreasing calcium levels some bands which appeared weak or absent in cystic saliva as compared to normal saliva reappeared. Conversely the electrophoretic appearance of normal saliva could be transformed into a pattern quite similar to that of cystic fibrosis by increasing calcium concentration to levels comparable to those in patients with cystic fibrosis. Studies with specific antisera showed no immunologic differences between the various bands while no immunologic differences were found between fractions from cystic or normal saliva. Chemical analysis of eluted bands showed glycoproteins to be major constituents.

It was concluded that submaxillary saliva in both normal subjects and patients with cystic firobsis contains as major constituents glycoproteins, probably identical, which combine or polymerize with calcium present in high concentrations to form a reversible precipitate of high molecular weight (>1,000,000) that cannot travel in the gel.

Immunological Studies of Submaxillary Saliva: It was demonstrated that in submaxillary saliva the following plasma proteins were present: prealbumin, albumin, alpha-antitrypsin, transferrin, and the three immunoglobulins, IgA, IgG, IgM; albumin and IgA being the main components. Orosomucoid, alpha_1-glycoprotein, ceruloplasmin, alpha_2-macroglobulin and fibrinogen could not be readily identified.

All protein fractions were present in similar amounts in submaxillary saliva from normal individuals and patients with CF. The only exception was IgA which was significantly elevated in submaxillary saliva as well as in the serum of patients with CF.

Ten specific components were identified in submaxillary gland tissue. One of them was shown to be alpha-amylase. Of the nine other tissue antigens found in submaxillary gland extracts, four were considered to be gland specific.

The submaxillary saliva antigen as well as the tissue antigens were demonstrated to be immunologically identical in the two groups and no antigen specific for CF was found.

Micropuncture Studies of Human Sweat Glands: Micropuncture and microanalytical methods were used in the investigation of the function of normal human eccrine sweat glands In order to determine whether the abnormality in sweat of cystic fibrosis patients is due to a dysfunction of the coil or of the duct osmolality was measured in fluid obtained by micropuncture from the coil of sweat glands of the nail fold in patients with cystic fibrosis. Osmolality of fluid from the coil was 323 mosm/±4.82 S.E. (40 measurements in 12 patients) which is not significantly different from that in normals. Therefore the abnormality in sweat formation in cystic fibrosis must be in the reabsorption of NaCl or water in the sweat gland duct rather than in the formation of the precursor solution in the sweat gland coil.

Electron-microscopic Studies of Sweat Glands: The Kommick pyroantimonate method for ultrastructural localization of sites presumably high in Na^+ was carried out on sweat gland biopsies of patients with cystic fibrosis and normal subjects before and after thermal sweating. Sweat gland secretory coil fixed with the osmic acid-$KSB(OH)_6$ solution revealed electron-dense deposits indicative of $NaSb(OH)_6$ aligned linearly on the mitochondrial cristae and the inner surface of the plasma membrane in the dark cells. No other sweat gland cell showed such deposits. Dark cells varied widely in the amount of such precipitate present. The cytoplasm of heavily reactive dark cells contained a fine diffuse precipitate outlining but not occupying secretory (carboxymucin-rich) vacuoles. Cytoplasm in weakly reactive dark cells and in light cells showed sparse coarse deposits throughout. Preliminary results indicate that mitochondrial precipitates are diminished although membrane precipitates are not necessarily changed.

Tamm-Horsfall Urinary Glycoprotein: It was shown that highly purified Tamm-Horsfall glycoprotein from both patients with CF and normal control subjects of the same age group had completely cross-reactive antibodies. The identity of the immunologic specificities of each was convincingly demonstrated by reciprocal absorption with each antigen, and in all cases, cross-absorption completely removed all reactions with either preparation. In addition, fragments prepared with tri-acetyl ammonium-bromide appeared to be immunologically identical in the two groups. All sugar components of the carbohydrate moiety of this glycoprotein as well as the ratio of fucose to sialic acid were similar in the two groups.

Significance to Bio-Medical Research and the Program of the Institute:

Investigations on Submaxillary Saliva: In these studies for the first time an attempt was made to assess the effect of abnormal ionic concentrations in CF on the solubility of glycoproteins in this disease. Since in CF widespread obstruction of organ passages gives rise to most of the pathologic and clinical findings, the polymerization of calcium with proteins to form a reversible precipitate of high molecular weight that cannot travel in acrylamide-gel might be one of the main mechanisms involved throughout the body.

In the immunologic studies much information was obtained as to composition of submaxillary saliva, not only in patients with CF but also in normal subjects, that was not previously available in the literature. The increase in IgA, the most important immunoglobulin of exocrine secretions, parallels that in the serum of patients with CF.

Studies of the Sweat Glands: The micropuncture studies of the precursor fluid in the sweat gland demonstrate for the first time by direct measurements that this solution is isotonic and identical with that of normal individuals. It therefore seems apparent that the eccrine sweat defect in CF must be due to a defect in reabsorption of the sweat gland duct.

Tamm-Horsfall glycoprotein investigations: The complete lack of difference in the Tamm-Horsfall glycoprotein from both patients with CF and control subjects is strong evidence against major structural changes in this glycoprotein fraction in cystic fibrosis.

Honors and Awards: None

Publications:

Grand, Spicer: Presumptive Ultrastructural Localization, of Na+ in the sweat gland in the Grindelwald Symposium. DiSant'Agnese, Talamo: New England Journal of Medicine. Schwartz, R. H. and Pallavicini, J.C. Tam-Horsfall Gugler, E., Pallavincini, Swerdlow, H., and di Sant' Agnese: The role of calcium in submaxillary saliva of patients with cystic fibrosis, J. Pediat. 1967. Gugler, et al of Pediat. (In press) Immunologic Studies Pallavicini J.C. Bruge Symposium.

Serial No: NIAMD:PMB:2c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effect of Flow Rate on Electrolytes and Proteins in Submaxillary Saliva of Normal Subjects and of Patients with Cystic Fibrosis

Previous Serial No: None

Principal Investigator: Paul A. di Sant' Agnese, M.D.

Other Investigators: Ulrich Wiesmann, M.D. (guest worker), Charles Pallavicini, PhD.; Stuart Handwerger, M.D.; Georges Peter, M.D.

Cooperating Units: NIDR, Dr. Swerdlow

Man Hours: Total: 1 3/4
Professional: 3/4
Other: 1

Project Description:

Objectives:

Saliva from the submaxillary gland offers an opportunity to study a relatively uncontaminated, glycoprotein-rich exocrine secretion in normal controls and in patients with CF. Since adequate amounts can be collected, a variety of organic and inorganic components can be analyzed in the same sample. In serial sampling over a long period of time, it is possible to study changes due to the varying rate of secretion as well as to exhaustion or fatigue of the gland, in both normal subjects and patients with cystic fibrosis.

Methods Employed:

Nine normal controls and nine patients with cystic fibrosis were used in these studies. Submaxillary saliva was collected with individually constructed appliancies. Serial samples of 2-4 ml were collected and from the volume the collection time of exact flow rates were calculated. Prior to stimulation 3 samples were collected. Then increasing amounts of 2% citric acid were applied on the tongue for the next 6-10 samples to stimulate increasing flow rates. The stimulation then was discontinued and another 3 samples collected.

Sodium, potassium, calcium and magnesium were determined by atomic absorption spectrophotometry. Inorganic phosphorus was measured colorimetically. Uric acid was assayed by the enzymatic method, total protein was determined by the Biuret reaction and ultra-violet absorption after enzymatic eliminations of uric acid. Polyacrylamide-gel and continuous flow electrophoresis were used to further define the single proteins at the various flow rates. IgA and albumin were quantitated by the immunodiffusion technique of Mancini. Total sugars and sialic acid were determined after acid hydrolysis spectrophotometrically. All of these determinations were made on each sample and at least 10 different experiments were performed on each normal control and on patients with CF over a period of three months up to several months. A total of more than 20,000 determinations were performed up to this time.

Major Findings:

1. Flow rates: A large variation in submaxillary maximal water output was observed in both patients and normals. However, while the pattern was similar in each individual normal subject during the whole period of observation in some patients with CF a progressive reduction of the water output over a period of several months was observed. In some of the cystic patients citric acid did not elicit any secretory activity, however, the submaxillary gland was still able to respond to stimulation with pilocarpine or methacholine. In others the glands were considered "dry" since by neither kind of stimulation significant amounts of secretion could be obtained. It should be mentioned that in such cases the secretion of parotid saliva was normal or even larger than average.

2. Electrolytes: The relationship of the concentration of electrolytes to the water output of the gland was established. It was shown that not only sodium and chloride are flow rate dependent, but also potassium, magnesium and calcium, although they respond in a different manner.

Although sodium rises with flow rate in linear fashion, in normal subjects two different types of responses were distinguished. Three individuals consistently secreted sodium at concentrations roughly double that of the other six subjects at comparable flow rates. In patients with CF submaxillary saliva sodium showed a marked vaiation between the different individuals, but three types of responses in sodium secretion were consistently present. If the figures for the two groups as a whole are considered, the sodium concentrations were not significantly different in normal subjects as compared with patients with CF. However, if the corresponding secretory groups are compared separately, the patients with CF show higher sodium concentrations than the corresponding normal subjects.

Potassium concentrations at very low flow rates up to 0.5 ml/min are inversely related to flow rate. At higher flow rates however the concentrations rise again, so that a U-shaped curve results. At the highest flow rates concentration tends to decrease again. The same type of curve can be found in cystics and in normals and no significant difference between these two groups was found in the potassium concentrations.

Magnesium concentrations also were inversely related to water output in both patients and normals. No significant difference was found between the concentrations of the two groups compared.

In normal subjects, calcium concentrations are high at very low flow rates and decrease with increasing flow rates. However, at flow rates higher than 0.5 ml/min the values increase again and at very high flow rates concentrations up to 6 mEq/L are found. In some patients with CF the concentrations of calcium were much higher and in some of them rose up to 15 mEq/L. However, other patients with CF had calcium concentrations within normal limits over the full range of flow rates.

A difference between submaxillary saliva from patients and from normal controls also was found in the concentrations of inorganic phosphorus. Phosphorus is also inversely proportional to flow rate. Starting from 25-30 mg% at low salivary flows the concentrations decreased exponentially in normal subjects.

3. Organic Constituents: Total hexoses, fucose and sialic acid concentration also increased with flow rate, but much less than in those of total protein. Most of the carbohydrates in submaxillary saliva are bound only to a few glycoproteins, which contain about 50% carbohydrates. However, no significant difference in concentration, ratio of fucose to sialic acid and to hexose, and in the relationship to flow rate could be found between normal subjects and patients with CF.

The so-called specific salivary protein, which are precipitated with calcium, are the ones which show the most striking increase with flow rate. At high salivary flows up to 15 times more of these proteins are excreted than in unstimulated low flow saliva. Again no overall difference between normals and patients could be observed. In previous studies we have shown that various serum proteins are present in submaxillary saliva, the two major fractions being albumin and γ globulin. On poly-acrylamide gel electrophoresis we could identify albumin in a band which was strong in low flow rate saliva and decreased in its strength with increasing flow. With increasing water output both albumin and IgA concentration decrease considerably and reach a constant plateau at a flow rate of approximately 1 ml/min. The shape of the curves are of the exponential type and similar for both proteins. The plateau concentration of IgA is roughly double that of the albumin concentration. Again there is little difference between the values from normal subjects and from patients with CF.

Uric acid behaves very much like the serum proteins. The initial concentrations in low flow rate saliva are around 6 mg%; the decrease is exponential and the values level out at 1-2 mg%. In saliva from cystics there is a delay in the decrease in the concentration of uric acid, similar to what was observed in inorganic phosphorus. Therefore, uric acid concentrations tend to be higher than in normals.

Significance to Bio-medical Research and the Program of the Institute:

These physiologic studies add considerably to our knowledge of cystic fibrosis. Heretofore, only the sweat gland had been studied from the standpoint of physiology, but not a mucous-producing exocrine gland. These investigations for the first time give an insight in the function of such a gland in this disorder. As in the case of the sweat gland the results in many instances were surprisingly normal. It appears as if some patients with CF are normal from the submaxillary standpoint regardless of involvement of other areas of the body. With the passage of time the submaxillary gland often appears to be involved in the process characteristic of this disease; the concentrations of protein and calcium then seem to rise as the flow rate decreases. It is at this point that calcium and proteins probably interact to form a precipitate which blocks the ducts and initiates a rapid worsening of the disease process. The submaxillary gland is presumably only a model of what happens in other areas in cystic fibrosis and causes pancreatic achylia, chronic pulmonary disease, hepatic cirrhosis and other obstructive complications.

Honors and Awards: None

Publications: None

Serial No: NIAMD:PMB:3c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of "Mucus" in Normal Human Submaxillary Saliva

Previous Serial Number: Same

Principal Investigator: J. Charles Pallavicini, Ph.D.

Other Investigators: Ulrich Wiesmann, M.D. (Guest worker)

Cooperating Units: None

Man Hours:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives:

This project was undertaken to clarify the physical-chemical relationships between proteins, glycoproteins, and electrolytes in normal human submaxillary saliva. Ultimate objective is to apply the knowledge thus acquired to the study of cystic fibrosis of the pancreas.

Methods Employed and Major Findings:

Reflex stimulated saliva was pressure filtered at room temperature through a 0.05μ average pore-size membrane. The effluent was non-viscous, usually clear and qualitatively contained most, if not all of the salivary proteins. On the membrane a white rubber-like layer was deposited. This material was dissolved in twice the original volume of water and centrifuged to remove a small amount of debris from the viscous filament-forming supernatant - Fraction 1. (Fraction 1 can be separated from saliva also by centrifugation at 3800 x g for 10 minutes either at 4° or 35°C. Evidence that its presence in saliva is not dependent upon the temperature condition of its isolation.) The supernatant, made to 40% - 50% ethanol was centrifuged to remove traces or remaining insoluble material and the supernatant made to 66% ethanol. After standing overnight at 4°C a precipitate was centrifuged down and dissolved with water to the original volume - Fraction 2. This fraction, though still viscous, had lost its filament-forming character.

Fraction 1 in immunodiffusion analysis gave precipitation line similar to those produced by total saliva, whereas Fraction 2 gave only one line. Chemical analysis of Fraction 2 showed 13.8% galactose, 19.8% fucose, 1.5% sialic acid, 7.9% hexosamine and 57.0% protein. Following acrylamide-gel electrophoresis of Fraction 2 at pH 8.4 a strong PAS reaction was observed only in the sample gel, while two distinct protein lines were visible in the running gel. If, prior to electrophoresis, Fraction 2 was treated with EDTA a pronounced additional protein band appeared in the running gel. This result suggests that some protein is associated in some manner with the Schiff-positive glycoprotein.

Significance to Bio-medical Research and the Program of the Institute:

It was concluded that in submaxillary saliva there is a complex system of proteins, glycoproteins and electrolytes. The glycoproteins are able to aggregate to very large units, which can be filtered and centrifuged and can bind proteins, presumably by calcium bridges, to form even larger complexes. Although the nature of this link is not fully understood the proteins appear to protect these large glycoprotein molecules against chemically-induced precipitation and denaturation.

Honors and Awards: None

Publications: Paper in press on this subject and with this title in the Brugge Symposium

Serial No: NIAMD:PMB:4c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Ekkrinosiderophilin of Human Milk

Previous Serial No: None

Principal Investigator: Arthur Schade, Ph.D., Laboratory of Microbiology

Other Investigators: Charles Pallavicini, Ph.D.; Ulrich Wiesmann, M.D.

Cooperating Units: LM:NIAID, (Dr. Schade); PMB:NIAMD

Man Hours:
Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives:

Ekkrinosiderophilin, first described in milk as lactosiderophilin, is known to occur in a variety of exocrine secretions including urine, where it seems to have a bacteriostatic effect. The physico-chemical properties of this red, iron containing protein have been investigated by different groups, but the findings have been contradictory. Our main objective was to study the physico-chemical properties of a very careful preparation of ekkrinosiderophilin and to compare it with the data from the literature.

Methods Employed and Major Findings:

Isolation of Ekkrinosiderophilin: Transitional human milk (5-10 days post parturition) was treated according to the procedure of Szuchet-Derechin and Johnson to obtain their so-called "albumin" fraction. This procedure involved the addition to whole milk of 20 g. sodium sulfate/100 ml milk followed by centrifugation to remove the cream, casein, and immunoglobulins. The clear straw-colored supernatant was then further salted with 20 g. ammonium sulfate/100 ml supernatant to precipitate the remaining protein including the ekkrinosiderophilin. The redissolved protein, equilibrated against 0.005-M phosphate buffer at pH 7.4 was applied to a DEAE Sephadex A-50 column similarly equilibrated. Following elution with 0.005-M buffer, a 0.05-M phosphate buffer at pH 7.4 was used. Two peaks of dλ280mμ-absorbing materials were obtained. The first proved to be that of ekkrinosiderophilin as indicated in the course of the run by an identical peak of Fe^{59} activity and by subsequent analyses.

Spectral absorption, visible and u.v.: When iron is added to decreamed skim milk, a salmon pink color develops similar to that shown by siderophilin of plasma and conalbumin of egg white. There is a broad peak of absorption at λ460-470mμ with a maximum at λ463mμ. Our DEAE-Sephadex purified iron-ekkrinosiderophilin (pH 7.0) absorbs maximally at the same wavelength and minimally at λ402mμ. The u.v. absorption curve shows a maximum at λ280mμ and a minimum at λ252mμ.

Electrophoretic Mobility: Although there is an apparent consensus that ekkrinosiderophilin behaves electrophoretically as a β protein, our investigations demonstrate that in filter paper, cellulose acetate, and polyacrylamide gel electrophoreses, the protein appears as a slow-moving γ globulin as compared to human serum and purified plasma siderophilin. We obtain this result whether we use skim milk, the "albumin" fraction, or the purified ekkrinosiderophilin. When the isolated ekkrinosiderophilin is subjected to immunoelectrophoresis, a single line of precipitation develops in proximity to the reservoir as does the $\beta 1$ globulin, plasma siderophilin. We wish to observe here that in polyacrylamide gel electrophoresis at pH 8.4 in borate buffer, ekkrinosiderophilin is found as fixed in the sample gel and at the beginning of the separating gel plus a varying fraction 1 to 2 cm in the separating gel of a total length of 15 cm. Roughly 80% of the total ekkrinosiderophilin is obtained in the separating gel when skim milk is the applied sample. As the milk is fractionated, first as "albumin" fraction and then as purified ekkrinosiderophilin, the percentage of mobile iron-binding protein progressively diminishes to less than 15% of the total. Thus, our isolated ekkrinosiderophilin as well as a sample generously supplied by Prof. Heremans in Belgium essentially did not move beyond the origin of the separating gel. Antiserum produced in rabbits by use of Prof. Heremans' material precipitated all of the iron-binding protein of skim milk and so indicates that both the immobile and mobile fractions are immunologically identical. Further, Ouchterlony tests show only one line of precipitation for the skim milk versus antiserum to the isolated ekkrinosiderophilin and again only one line of precipitation for the isolated ekkrinosiderophilin versus the antiserum to skim milk.

Sedimentation and Molecular Weight of Ekkrinosiderophilin: Employing the sucrose gradient technique of Martin and Ames, we determined the $S_{20,w}^{0.725}$ of the iron-binding component in skim milk by comparison with catalase and plasma siderophilin. An $iron^{59}$ tag was used to locate both iron-chelating moieties. The results obtained from which the $S_{20,w}$ of the milk iron-binding component was calculated to be 8.7 - 8.9. When the isolated ekkrinosiderophilin was similarly run versus catalase, an average $S_{20,w}$ value of 5.3 was given by this method. This latter value compared reasonably with an $S_{20,w}$ value of 4.68 determined by ultracentrifugation analysis of the same ekkrinosiderophilin. The significantly different S value for the iron-binding component is skim milk from that shown by the isolated ekkrinosiderophilin strongly suggests that in milk the ekkrinosiderophilin is associated with another protein or exists in other than monomeric form. Confirmation of such association was observed when the isolated ekkrinosiderophilin was mixed with skim milk and the mixture analyzed in the sucrose gradient setup. A control was run of the ekkrinosiderophilin in 5% human albumin in place of milk. The ekkrinosiderophilin added to skim milk sedimented at the same rate as the iron-binding component in the milk ($S_{20},w = 8.7$-8.9) while that added to the albumin sedimented at the slower rate ($S_{20},w = 5.3$).

Employing the determined S value of 5.33 for our purified ekkrinosiderophilin for 0.5% concentration in 0.05-M phosphate buffer at pH 7.5 and 25°C and the diffusion constant of 6.0 x 10-7, assuming a partial specific volume of 0.73, we calculated the molecular weight to be 82,500.

Honors and Awards: None

Publications: Schade, A.L., Pallavicini, C., and Wiesmann, U.: Ekkrinosiderophilin of Human Milk in Protides of the Biological Fluids: Proceedings of the Sixteenth Colloquium, Bruges 1968. (In press)

Serial No: NIAMD:PMB:5c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Albumin Turnover Studies in Cystic Fibrosis of the Pancreas (CF).

Previous Serial Number: Same

Principal Investigator: Georges Peter, M.D.

Other Investigators: Stuart Handwerger, M.D.; Paul A. di Sant' Agnese, M.D.

Cooperating Units: W. Strober, M.D.; T. Waldmann, M.D. Metabolism Unit, NCI

Man Hours:	Total:	3/4
	Professional:	3/4
	Other:	None

Project Description:

Objectives:

Hypoalbuminemia in cystic fibrosis occurs most commonly in moderately or severely ill patients with advanced pulmonary disease. In spite of this finding, its cause has not been elucidated. In view of the frequency of malabsorption, hepatic involvement, and cor pulmonale in cystic fibrosis, hypoalbuminemia in this disease could result from hyposynthesis due either to malabsorption of amino acids or to liver disease; hypercatabolism of protein secondary to protein-losing enteropathy; and/or dilution of a normal albumin pool in an abnormally large space of distribution such as in congestive heart failure. The previous study, on the basis of data from three patients, suggested the latter cause.

Methods Employed and Major Findings:

22 ^{125}I albumin turnover studies were performed on 16 patients with cystic fibrosis, including 10 patients with hypoalbuminemia. Nineteen ^{51}Cr albumin turnover studies were performed on seventeen patients to quantitate gastro-intestinal protein loss. The data was analyzed according to a modification of the methods of Berson. The ^{51}Cr albumin data was expressed both as gastro-intestinal clearance and as four day fecal excretion of ^{51}Cr.

Major Findings:

The plasma volumes of the hypoalbuminemic patients were significantly greater than those of the normoalbuminemic and control subjects. Mean plasma volume of the patients with low serum albumin (57.4±9.4cc/kg) was significantly greater (p value <0.01) than the corresponding mean of the normoalbuminemic patients (43.6±3.1cc/kg). Similarly, 13 or 16 studies of hypoalbuminemic patients demonstrated hypervolemia in contrast to none of the patients with normal serum albumin concentrations. In contrast, the mean total circulating albumins, albumin synthetic rates, fractional catabolic rates, and per cent IV albumin of the hypoalbuminemic patients, normoalbuminemic patients, and controls were similar.

Two patients with CF, however, did have albumin synthetic rates below the normal range and both had hepatic cirrhosis. The four other patients with only laboratory evidence of hepatic involvement who were studied demonstrated normal albumin synthetic rates.

The ^{51}Cr albumin studies demonstrated that the majority of patients had slightly increased 4 day fecal excretion of protein and gastro-intestinal clearance of ^{51}Cr albumin. The incidence of increased G.I. protein loss was higher among the hypoalbuminemic patients but calculations of the effect of this loss upon serum albumin indicated that this factor was only of minor importance in the pathogenesis of hypoalbuminemia.

Thus, hypoalbuminemia in the presence of advanced pulmonary disease in cystic fibrosis was shown to result primarily from hypervolemia. Clinical and post mortem data established that this hypervolemia probably is secondary to cor pulmonale. In addition, decreased albumin synthesis can, but does not usually, result from the hepatic involvement of cystic fibrosis.

Significance to Bio-medical Research and the Program of the Institute:

The study represents the first comprehensive and careful investigation of albumin metabolism and hypoalbuminemia in cystic fibrosis. It is apparent from the results reported that a low serum albumin in patients with CF and severe pulmonary disease is usually due to hemodilution because of increased blood volume secondary to cor pulmonale. Although directly related to the severity of the pulmonary disease, hypoalbuminemia may appear days or months before it is clinically apparent that cardiac involvement is present and as such may be a simple and invaluable guide to prognosis and treatment in this disease.

Honors and Awards: None

Publications: Strober, W., Peter, G., Schwartz, R.: Albumin Metabolism in Cystic Fibrosis: (Pediatrics, in press)

Serial No.NIAMD:PMB:6c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Carbohydrate Metabolism in Cystic Fibrosis of the Pancreas

Previous Serial Number: NIAMD-PMB-5c

Principal Investigator: Stuart Handwerger, M.D.

Other Investigators: Paul A. di Sant' Agnese, M.D.; Georges Peter, M.D.

Cooperating Units: Drs. Jesse Roth, Philip Gorden, Clinical Endocrinology Branch, NIAMD; John Edgcomb, Deborah Carpenter, Pathologic Anatomy Branch, NCI.

Man Hours:
Total: 3/4
Professional: 3/4
Other: 0

Project Description:

Objectives:

Glycosuria and abnormal glucose tolerance test have been seen the increasing frequency as the life span of patients with CF is becoming more prolonged and a number of adolescents and young adults with this disease have been observed. In the past, this intolerance has been attributed to classical diabetes mellitus, although there were many reasons to doubt this conclusion.

This project was undertaken with a four-fold objective: 1. further characterize the etiology of the glycosuria and glucose intolerance; 2. assess the incidence of this complication; 3. better understand its clinical course; and 4. to determine the best therapeutic approach.

Methods Employed:

Patient material consisted of thirty-one patients with cystic fibrosis, 7 to 28 years of age, as well as four patients with recognized diabetes mellitus with insulinopenia were studied. Ten normal volunteers served as controls.

Tests consisted of oral glucose tolerance test - 1.75 grams per kg. actual body weight up to 100 grams was used. Intravenous glucose tolerance test - 0.5 grams glucose per kg. actual body weight up to 25 grams was used. Insulin tolerance test - 0.1 unit regular porcine insulin per kg. actual body weight was given intravenously. Glucose - tolbutamide test. Oral glucose (as above) given and, two hours later, 1 gram of tolbutamide was infused. Glucose-glucagon tolerance test - oral glucose given as above and, two hours later, an intravenous infusion of glucagon, 6 gamma per minute, was started.

Glucose was measured by the auto-analyzer. Serum insulin and growth hormone was determined by double antibody radio-immunoassays.

Major Findings:

Oral glucose tolerance tests were performed on 31 patients (median age 17). Fifty-eight per cent had normal or nearly normal glucose tolerance, thirty-two per cent had moderate glucose intolerance, and 10 per cent had severe glucose intolerance with elevated fasting glucose concentrations. However, regardless of glucose tolerance, all patients had a decreased total output of insulin. Patients with normal or nearly normal GTT reached peak insulin levels at one hour as did normal subjects. Patients with moderate glucose tolerance, however, did not reach peak insulin values until 2 hours. In patients with severe glucose intolerance, insulin levels did not rise appreciably above fasting concentrations despite marked hyperglycemia.

Blood glucose responses following intravenous glucose were less impaired than following oral glucose. Nevertheless, insulin secretion was diminished in all patients and peak insulin levels were frequently delayed.

Glucose, tolbutamide and glucose-glucagon tests, however, revealed that tolbutamide and glucagon could stimulate further increases in serum insulin concentrations when given at the height of the insulin response to oral glucose. A repeat dose of oral glucose did not cause appreciable increases in insulin levels. These results suggest that the decreased insulin secretion to glucose resulted from a defect in the mechanism whereby glucose stimulates insulin release.

Insulin tolerance tests were normal regardless of glucose tolerance. Growth hormone secretion to exogenous insulin was also normal. Phentolamine, and alpha adrenergic blocker, failed to alter insulin secretion.

Clinically, only the patients with glycosuria had symptoms and tolbutamide was usually sufficient for the treatment of these patients.

Classical diabetes mellitus was unlikely as a cause of the defect in our patients. In contrast to juvenile diabetics, CF patients with glucose intolerance 1. usually had a negative family history for diabetes, 2. generally had a benign clinical course, 3. seldom required treatment, 4. had no evidence of retinopathy, nephropathy, or neuropathy, and 5. had no vascular changes at autopsy. Furthermore, juvenile diabetics failed to increase their insulin concentrations after tolbutamide and glucagon.

Pathologic examination of the islets of Langerhans in over 35 patients, including seven patients in this study, was performed. Islet tissue was only modestly decreased but fibrosis was noted in most islets. This fibrosis separated beta cells from each oter, from adjacent alpha cells, and from neighboring blood vessels. We feel that this fibrosis, by altering the synergistic effect of the agents known to augment insulin secretion to glucose, could possibly explain the glucose intolerance in our patients. However, verification will have to await a better understanding of the normal mechanisms of insulin secretion.

Significance to Bio-medical Research and the Program of the Institute:

This represents the first comprehensive investigation of this important complication of CF using the techniques for radio-immuno-assay of serum insulin levels as well as pathologic studies. It was demonstrated that the glycosuria which has been seen increasingly with the advancing age of CF patients in recent years is not due to classical diabetes mellitus, but is due to disruption of the islet architecture because of the increasing fibrosis of the pancreas. These conclusions have an important bearing on the pathogenesis and treatment of cystic fibrosis. They may also influence medical thinking in the evaluation of so-called fibrosis such as chronic recurrent pancreatitis.

Honors and Awards: None

Publications: In press

CLINICAL HEMATOLOGY BRANCH

I. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

1. Platelet metabolism.

Following the demonstration in collaboration with Drs. Warshaw and Laster that platelets formed new protein *in vitro*, we have in the past year attempted to define the nature of the proteins formed and the physiologic circumstances responsible for variations in rate of protein synthesis. Various proteins in platelet sap have been separated by a variety of techniques including column chromatography, gel filtration, and immunoelectrophoresis. Of special interest are macromolecular proteins with fibrinogen-like characteristics which appear to be labeled with radioactivity when radioautographs are made of immunoelectrophoretic and gel diffusion patterns. These proteins are considered unique to platelets and involved in their clotting and clot retraction functions. We have experienced difficulty in separating the fibrinogen-like protein from the retractile protein of platelets, called thrombosthenin; for both cross-react immunologically. The literature suggests these proteins can be clearly separated but we doubt it.

We have found that protein formation in platelets is associated with only young platelets and that platelets that are hemostatically active are probably the ones actively forming proteins, whereas the older platelets which account for perhaps 75% of those circulating are relatively inactive both metabolically and hemostatically.
(Drs. N. Raphael Shulman and Ariana B. Students).

2. Fibrinolysis.

Following the qualitative identification of two intermediate fibrin degradation products formed very early in the process of fibrinolysis, prior to formation of the final products, further studies have been performed to identify the intermediate split products by their physicochemical characteristics and biochemical reactions. The earliest split product which we have labeled "Fragment X" was found to have ultracentrifugal sedimentation characteristics at higher concentrations that overlap those of native fibrinogen owing possibly to the tendency of the split product to form more dimer and trimer complexes compared to native fibrinogen. This finding explains the inability of other workers to clearly identify the early split product by sedimentation techniques, and accounts for reports in the literature of peculiar forms of "light" fibrinogen found under some circumstances of fibrinogen purification. The molecular characteristics of the fibrinogen molecule have been further defined by the finding that the antigenic determinants of the X and Y fragments described last year are found in subsequent degradation products A, B, C, D, and E. Quantitative recovery of the fragments obtained from the intermediate degradation products permitted establishing a hypothetical model of fibrinogen structure based on relationships between the proteolytic fragments. Interaction between the fragments and fibrinogen was found to be the basis for their anticoagulant activity. Quantitative relationships between reactants in the anticoagulant complexes were defined. (Drs. Victor J. Marder, N. Raphael Shulman, and William R. Carroll (LBC)).

3. Physiologic control of Factor VIII levels.

Following our previous finding that the spleen has an important role in the physiologic control of Factor VIII levels, further investigations have been carried out to determine whether the spleen manufactures Factor VIII, controls manufacture elsewhere by a humoral mechanism, and/or stores Factor VIII. These studies which involve surgical manipulations such as hepatectomy, splenectomy, portacaval shunts, and combinations of these various procedures, done in collaboration with Dr. William G. Hammond of the Cancer Institute, indicate that the spleen is not important in manufacturing physiologic amounts of Factor VIII, that the liver is the most likely site of major amounts of Factor VIII production, and that the reticuloendothelial system may be a major site of Factor VIII destruction. These findings indicate that the current pressure in some medical centers to perform splenic transplants for correction of hemophilia is unwarranted.
(Drs. Stanley P. Watkins, Jr., Samuel B. Itscoitz, N. Raphael Shulman, and William G. Hammond (SURG, NCI)).

4. Factor VIII inhibitors.

In the past five years we have seen twelve patients with an acquired anti-Factor VIII that is antibody in nature. All the antibodies so far are 7S and all but two react with Factor VIII from a variety of species. Two did not react with bovine or porcine Factor VIII and the patients could be treated effectively with Factor VIII made from these animals if necessary.

Some reports have appeared in the literature suggesting that the spontaneously arising anti-Factor VIIIs are monoclonal in nature, for only the kappa type of gamma globulin was found to account for the anticoagulant activity. We have found that the anti-lambda antibodies available for these studies are generally too weak to inactivate most antibodies with characteristics of the anti-Factor VIII present in these patients. Rather than monoclonal "autosensitivity" antibodies owing to mutant lymphocytes, their activity simply may be the result of a cross-reaction.
(Drs. N. Raphael Shulman, Richard J. Hirschman, and Victor J. Marder).

5. Evidence that the spleen retains the youngest and hemostatically most effective platelets.

When platelet levels were depressed in normal individuals by plasmapheresis there was a lag in return of platelets for approximately 2 days, then a rise of 10 to 15% per day to control levels. Splenectomized individuals made similarly thrombocytopenic showed no lag in return of platelets and reached control values approximately 2 days earlier. Platelets transfused from splenectomized into normal individuals remained at a constant

concentration in the recipient for 2 to 3 days before disappearing linearly in the normal fashion over a 10-day period. These findings suggest that newly formed platelets are sequestered in the spleen for approximately 2 days before being released into the circulation. That young and old platelets are distinguishable is evident from their metabolic activities, for platelets up to 3 days old (obtained from patients splenectomized to correct thrombocytopenia) synthesized 4 to 5 times more protein than did platelets from normal individuals. Hemostatic properties of young and old platelets were assessed by transfusing patients severely thrombocytopenic due to a production defect with sufficient platelets to provide a normal level (> 150,000 and up to $600{,}000/mm^3$) for more than 5 days after infusion. The patients' bleeding times, initially corrected to normal, became infinite 4 or 5 days after transfusion of platelets obtained from splenectomized donors, and earlier when normal donors were used. Thus, platelets are functional for a maximum of 4 to 5 days after production. Since platelets apparently reside for approximately 2 days in the spleen, their hemostatic activity in the circulation lasts for only 2 or 3 days of their 10-day survival. Thus, the total number of platelets circulating was found to be less important than platelet age in promoting hemostasis. These findings explain for the first time the significance of the bleeding time test and the paradoxical differences in susceptibility to hemorrhage and responses to therapy that occur in thrombocytopenic disorders of different etiology.
(Drs. N. Raphael Shulman, Stanley P. Watkins, Jr., Samuel B. Itscoitz, and Ariana B. Students).

II. Study of Immunology of Blood Cell Deficiencies

1. Idiopathic thrombocytopenic purpura.

We have continued our analysis and classification of sub-groups of disorders within the inclusive syndrome of ITP by measuring the plasma level of anti-platelet (or ITP) factor and determining megakaryocyte function by specific activity changes of Cr^{51}-labeled platelets in an additional 12 cases of ITP (total 35). Approximately 20% of the cases appear to be caused by megakaryocyte failure or ineffective thrombopoiesis rather than ITP factor. Patients with an anti-platelet factor respond to treatment with adrenocorticosteroids and splenectomy in a manner that can be predicted by the titer of ITP factor, whereas those caused by abnormal thrombopoiesis do not respond to any current form of therapy.
(Drs. N. Raphael Shulman, Stanley P. Watkins, Jr., Samuel B. Itscoitz, and Ariana B. Students).

2. Platelet and leukocyte isoantigens.

We have continued to identify new specificities of isoantigens that are considered to be transplantation antigens. Our panel of antibodies has been used to cross match donor-recipient pairs for 40 renal transplants retrospectively, and information derived by comparing longevity of the graft as well as histologic changes in the graft indicate that most, if not all, of the leukocyte isoantibodies available to us for cross-matching are not valuable in predicting rejection of a transplant.

In collaboration with Drs. Grollman and Ginsburg, we have attempted to inhibit the reactions between specific leukocyte isoantibodies and their antigens by potentially haptenic sugars derived from human milk. These sugars, which were found by Drs. Grollman and Ginsburg to inhibit some erythrocyte antigen-isoantibody reactions, did not inhibit any of the anti-platelet or anti-leukocyte isoantibodies. Inhibition studies of this type, if found to be effective in vitro, may provide an approach to therapy of transplantation rejection phenomena.

A variety of long-term cultured leukocyte cells derived from normal individuals and patients with lymphoma, leukemia, and infectious mononucleosis have been evaluated for their content of isoantigens, both qualitatively and quantitatively. These cells have been found to retain their isoantigenic characteristics over at least several years in culture. In some instances there has been a unique opportunity to compare the antigenicity of the cells from the living donor with that of long-term cultured cells, and no change in quantity or quality of antigen per cell has been noted. These findings are of importance in identifying cultured cell lines, in studying transplantation phenomena in vitro, and in immunologic cancer chemotherapy studies.
(Drs. N. Raphael Shulman, Evelyn Grollman (LBP), Victor Ginsburg (LBP), Yashar Hirshaut (M, NCI), J. Moor-Jankowski (New York University), and S. A. Mayyasi (Smith Memorial for Cancer Research, Pfizer & Co., Inc.).

3. Transplantation antigens of non-human primates.

The group of chimpanzees and baboons we immunized in previous years are now valuable animals used by at least four groups of investigators to study erythrocyte, leukocyte and platelet antigen systems. In the past year, these animals were given booster injections of human and non-human primate cells to maintain their antibody titers. Continued study of the specificity of chimpanzee leukocyte and platelet blood groups has resulted in identification of several new antigens during the past year, two of which are identical to human antigens. Dr. Lalazeri of New York University and Drs. van Rood and Balner of the University of Leiden, Netherlands, have gone to Holloman Air Force Base to study our animals to compare their antigens and antisera with the human systems they have defined.
(Drs. N. Raphael Shulman, J. Moor-Jankowski (New York University), Clyde Kratochvil (Holloman Airforce Base, New Mexico), and Miss Merilyn Hiller).

4. Drug antibodies.

In collaboration with Dr. Louis Sargent, quinine and quinidine were diazotized to a variety of human and animal proteins and these haptene protein complexes were evaluated quantitatively in reactions with antibodies against quinine and quinidine that arise clinically. It was found that the bound drugs react as effectively as free drugs in attaching antibodies

to platelets. The availability of a macromolecular form of antigen provides a basis for developing a sensitive, simple clinical test for these antibodies that may bypass the use of a blood cell.
(Drs. N. Raphael Shulman, Louis Sargent (LC), and Miss Merilyn Hiller).

5. Isoimmune neonatal purpura.

Serum from otherwise normal mothers whose infants are thrombocytopenic continues to be an excellent source of anti-platelet and anti-leukocyte isoantibodies. Twenty additional mothers have been studied this year. This continued documentation of the isoantigenic specificity of cells that sensitize mothers will be helpful in establishing the relative antigenicity of the types of antigens that are generally considered to be significant in histocompatibility.

In addition, continued documentation of response of thrombocytopenic infants to adrenocorticosteroid and exchange transfusion therapy with respect to maternal isoantibody type and titer will be helpful in establishing the most effective form of treatment for the disorder.
(Dr. N. Raphael Shulman, and Miss Merilyn C. Hiller).

Serial No. NIAMD-CHB-1c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis.

Previous Serial Number: SAME

Principal Investigator: Dr. N. Raphael Shulman

Other Investigators:
Dr. Richard J. Hirschman
Dr. Samuel B. Itscoitz
Dr. Victor J. Marder
Dr. Ariana B. Students
Dr. Stanley P. Watkins, Jr.

Cooperating Units:
Dr. William R. Carroll, LBC, NIAMD
Dr. William G. Hammond, SURG, NCI

Man Years
Total: 5-1/4
Professional: 4-1/4
Other: 1

Project Description:

Objectives:

Study of the reactions and interactions of coagulation factors *in vitro* and *in vivo* to define further the nature of the blood coagulation mechanism, and to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis and develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactively labeled substrates.

Major Findings:

1. Platelet metabolism.

Following the demonstration in collaboration with Drs. Warshaw and Laster that platelets formed new protein *in vitro*, we have in the past year attempted to define the nature of the proteins formed and the physiologic circumstances responsible for variations in rate of protein synthesis. Various proteins in platelet sap have been separated by a variety of techniques including column chromatography, gel filtration, and immunoelectrophoresis. Of special interest are macromolecular proteins with fibrinogen-like characteristics which appear to be labeled with radioactivity when radioautographs are made of immunoelectrophoretic and gel diffusion patterns. These proteins are considered unique to platelets and involved in their clotting and clot retraction functions. We have experienced difficulty in separating the fibrinogen-like protein from the retractile protein of platelets, called thrombosthenin; for both cross-react immunologically. The literature suggests these proteins can be clearly separated but we doubt it.

We have found that protein formation in platelets is associated with only young platelets and that platelets that are hemostatically active are probably the ones actively forming proteins, whereas the older platelets which account for perhaps 75% of those circulating are relatively inactive both metabolically and hemostatically.

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Following the qualitative identification of two intermediate fibrin degradation products formed very early in the process of fibrinolysis, prior to formation of the final products, further studies have been performed to identify the intermediate split products by their physicochemical characteristics and biochemical reactions. The earliest split product which we have labeled "Fragment X" was found to have ultracentrifugal sedimentation characteristics at higher concentrations that overlap those of native fibrinogen owing possibly to the tendency of the split product to form more dimer and trimer complexes compared to native fibrinogen. This finding explains the inability of other workers to clearly identify the early split product by sedimentation techniques, and accounts for reports in the literature of peculiar forms of "light" fibrinogen found under some circumstances of fibrinogen purification. The molecular characteristics of the fibrinogen molecule have been further defined by the finding that the antigenic determinants of the X and Y fragments described last year are found in subsequent degradation products A, B, C, D, and E. Quantitative recovery of the fragments obtained from the intermediate degradation products permitted establishing a hypothetical model of fibrinogen structure based on relationships between the proteolytic fragments. Interaction between the fragments and fibrinogen was found to be the basis for their anticoagulant activity. Quantitative relationships between reactants in the anticoagulant complexes were defined.

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Honors and Awards: None

Publications:

1. Shulman, N. R., Cowan, D. H., Libre, E. P., Watkins, Jr., S. P., and Marder, V. J.: The physiological basis for therapy of classic hemophilia (Factor VIII deficiency) and related disorders. National Institutes of Health Combined Clinical Staff Conference. Ann. Intern. Med. 67:856-881, Oct. 1967.

2. Cooke, J. V., Holland, P. V., and Shulman, N. R.: Cryoprecipitate concentrates of Factor VIII for surgery in hemophiliacs. Ann. Intern. Med. 68:39-47, Jan. 1968.

3. Shulman, N. R.: Surgical care of patients with hereditary disorders of blood coagulation. Modern Treatment 5:61-83, Jan. 1968.

4. Libre, E. P., Cowan, D. H., Watkins, Jr., S. P., and Shulman, N. R.: Relationships between spleen, platelets and Factor VIII levels. Blood 31:358-368, Mar. 1968.

5. Marder, V. J., Shulman, N. R., and Carroll, W. R.: The importance of intermediate degradation products of fibrinogen in fibrinolytic hemorrhage. Trans. Assoc. Am. Phys. 80:156-167, 1967.

6. Larrieu, J. J., Inceman, S., and Marder, V.: Action des produits de degradation du fibrinogene sur les fonctions plaquettaires. Nouv. Rev. Franc. d'Hematol. 7:691-704, 1967.

7. Marder, V. J.: Immunologic structure of fibrinogen and its plasmin degradation products: Theoretical and clinical considerations. In Laki, K. (Ed.): Fibrinogen. New York, Marcel Dekker, Inc., 1967, pp. 339-358.

Serial No. NIAMD-CHB-2c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of the Immunology of Blood Cell Deficiencies

Previous Serial Number: SAME

Principal Investigator: Dr. N. Raphael Shulman

Other Investigators: Dr. Richard J. Hirschman
Dr. Samuel B. Itscoitz
Dr. Victor J. Marder
Dr. Ariana B. Students
Dr. Stanley P. Watkins, Jr.

Cooperating Units: Dr. Evelyn Grollman, LBP, NIAMD
Dr. Victor Ginsburg, LBP, NIAMD
Dr. Louis Sargent, LC, NIAMD
Dr. Yashar Hirshaut, M, NCI
Dr. J. Moor-Jankowski, New York University
Dr. Clyde Kratochvil, Holloman Airforce Base, New Mexico
Dr. S. A. Mayyasi, Pfizer & Co., Inc.

Man Years
Total: 5-1/4
Professional: 4-1/4
Other: 1

Project Description:

Objectives:

To study the nature of immunologic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homo-transplantation and in possibilities of developing immunologic therapy against malignancies.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physicochemical characterization of purified antibodies and antigens, micro-analyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutination and precipitin reactions, immuno-electrophoresis, methods of provoking antibody responses in man and animals, and isotopic and fluorescent labeling applied to antigens and antibodies. Methods of assessing reticuloendothelial function through clearance of labeled particles and organ localization of sequestered cells by external body counting. Tissue culture techniques, red cell agglutination techniques and lymphocyte transformation tests, including radioautography.

Major Findings:

1. Idiopathic thrombocytopenic purpura.

We have continued our analysis and classification of sub-groups of disorders within the inclusive syndrome of ITP by measuring the plasma level of anti-platelet (or ITP) factor and determining megakaryocyte function by specific activity changes of Cr^{51}-labeled platelets in an additional 12 cases of ITP (total 35). Approximately 20% of the cases appear to be caused by megakaryocyte failure or ineffective thrombopoiesis rather than ITP factor. Patients with an anti-platelet factor respond to treatment with adrenocorticosteroids and splenectomy in a manner that can be predicted by the titer of ITP factor, whereas those caused by abnormal thrombopoiesis do not respond to any current form of therapy.

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4. Drug antibodies.

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In addition, continued documentation of response of thrombocytopenic infants to adrenocorticosteroid and exchange transfusion therapy with respect to maternal isoantibody type and titer will be helpful in establishing the most effective form of treatment for the disorder.

Honors and Awards: None

Publications:

1. Shulman, N. R.: Nature and Distribution of Platelet Blood Groups. In Kuhns, W. J. (Ed.): Conference on Blood Groups and Blood Transfusion Vol. 1. New York, Better Bellevue Association, sponsored by the John A. Hartford Foundation, 1967, pp. 127-135.
2. Hiller, M. C., and Shulman, N. R.: Characteristics of leukocyte and platelet antigens shared by nonhuman primates and man. Ann. N. Y. Acad. of Sci., In press.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

OFFICE OF SCIENTIFIC COMMUNICATION

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Conference on Progress in Methods of Bone Mineral Measurement and Proceedings

Conference on the Nutritional Aspects of Uremia and Proceedings

First Annual Contractors' Conference: Artificial Kidney Program and Proceedings

Proceedings of Conference on Natural and Synthetic Membranes

Guide to Nutrition Terminology Project

SUMMARY

The Office of Scientific Communications is charged with the general programming and planning of communication activities concerned with the various areas under the purview of the NIAMD. The present activities can be roughly divided into four main categories:

1. Current awareness literature services;
2. Retrospective literature studies;
3. Conferences and workshops; and
4. Other aspects of communication, such as the production of a motion picture, and thesauri development.

In the area of current-awareness literature services, the Arthritis and Rheumatic Diseases Abstracts is the project of longest standing. It was initiated as a result of the need for and interest in such a publication by the leading rheumatologists in this country, particularly those in close contact with the Institute, such as council members, study section members, and grantees. This publication has been very well received and at the present time is in its fourth volume.

Much the same type of stimulus was involved in the establishment in January 1966 of Gastroenterology Abstracts and Citations, in 1967 of the Artificial Kidney Bibliography, and in 1968 of the Endocrinology Index (Bureau of the Budget clearance pending at this writing). The Gastroenterology Abstracts and Citations is now in its third volume and is expected to continue in much the same form that it now has. The Artificial Kidney Bibliography at present is limited to citations. Some revisions are contemplated in order to add abstracts and to more effectively cover the engineering and physical and chemical science aspects. Endocrinology Index is limited to citations, but is arranged in five sections which interdigitate, and consequently provide more extensive information to the user than does the Artificial Kidney Bibliography. The publication covers all of endocrinology except pancreatic function, covered by Diabetes Literature Index. It is prepared with the cooperation of the National Library of Medicine, through use of the Medical Literature Analysis and Retrieval System (MEDLARS).

Diabetes Literature Index was undertaken as a regular monthly publication in 1966 and is a byproduct of research undertaken under a complex of grants to three universities--Minnesota, Rochester, and Western Reserve. The Index will continue in its present form through 1967, but changes and improvements are planned on an experimental basis in 1968 as a result of further research and response from its users.

Another project concerns itself with retrospective literature searching, the Nutritional Requirements of Vertebrates Other Than Man, in which the NIAMD supports the activities of an NAS-NRC committee and its subcommittees in their efforts to maintain a constant vigil and review of the literature in regard to nutritional requirements of vertebrates and factors which directly influence these requirements. The publications which are a result of the endeavors of this committee are published by the National Academy of Sciences.

The management of conferences is also a responsibility of the Office of Scientific Communications. This effort may take any one of several forms--from taking the responsibility for the entire conference, including publication of the proceedings, to assuming responsibility for only one aspect of the conference, such as the publication of the proceedings. During the past year this office has been involved in the management of four conferences or workshops covering various subject areas of interest to the NIAMD, e. g., osteoporosis, bone mineral measurement, uremia and the Artificial Kidney Program. The proceedings of three of these conferences are being published. In addition, the proceedings, only, of two other conferences are being published by NIAMD.

The film dealing with the current methods of diagnosis and therapy of cystic fibrosis (described in the 1967 annual report) was completed and has been deposited for distribution with the Audio Visual Center in Atlanta, Georgia. The National Cystic Fibrosis Research Foundation which co-sponsored the film also has copies for distribution.

During the past year, a project co-sponsored by NICHD, was initiated with Vanderbilt University; Dr. E. Neige Todhunter, principal investigator for the preparation of a guide to nutrition terminology.

In addition, numerous other activities which bear on scientist-to-scientist communication as it affects the Institute are dealt with frequently.

Areas where further emphasis should possibly be placed are continually in review. Future activities may involve some expansion of the communication effort in the Artificial Kidney Program and the support of a film dealing with diagnosis and treatment of chronic renal failure.

Project Title: Arthritis and Rheumatic Diseases Abstracts

Contract Number: PH43-68-620

Amount: $56,245

Contractor: Excerpta Medica Foundation

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Dr. John Magill

Man Years: Total: 1.0
Professional: .25
Others: .75

Project Description:

Arthritis and Rheumatic Diseases Abstracts is a monthly current-awareness journal published by the Office of Scientific Communication, NIAMD. It was initiated in November 1964 and has been particularly well received. Now in its fourth volume, it contains each month approximately 250 abstracts of the current world literature pertaining to this field. It is distributed, free upon request, to qualified investigators and practitioners in the field of rheumatology as well as other government agencies with responsibilities in this area, medical schools, and hospital libraries. Cumulated subject and author indexes appear at the end of each volume. Beginning with the fourth volume, the subject index was changed from a conventional index to a conceptual computerized product. In addition to the free distribution of this publication to qualified investigators in the field, it is also for sale by the Superintendent of Documents, Government Printing Office.

Project Title: Diabetes Literature Index

Contract Number: PH43-67-663

Amount: $56,500

Contractor: University of Minnesota

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Mrs. Ethaline Cortelyou

Man Years: Total: .70
Professional: .32
Others: .38

Project Description:

Diabetes Literature Index, a monthly current-awareness publication of the NIAMD was brought about through the cooperative efforts of NIAMD, NLM, the American Diabetes Association, and three Universities (University of Minnesota, University of Rochester, and Western Reserve University), as well as a number of individual scientists. Preliminary study of computerized selective retrieval of diabetes-related literature citations was supported by a grant from the NIAMD, and in January, 1966 the monthly publication of the Diabetes Literature Index was begun under the above contract.

Each month MEDLARS magnetic tapes, which contain all of the current biomedical literature citations used for the preparation of the NLM's Index Medicus, are used for identification of all diabetes-related bibliographic citations.

For all of the diabetes-related literature chosen, the authors and key words in the stored titles are identified by the computer and an expanded magnetic tape is prepared, listing the complete bibliographic citation under each author and key word. These are alphabetized by the computer under both key words and authors, assembled into the appropriate page format, and printed by the computer. This computer output is then assembled into page format, ready to be photographed and used for offset printing. During the present volume a new format will be tried experimentally and user response sought.

Diabetes Literature Index is published monthly by the Office of Scientific Communication, NIAMD, and is available, free upon request, to qualified individuals working in the field of diabetes research as well as to other government agencies with responsibilities in this area, medical schools, and hospital libraries. In addition to the free distribution of this publication to qualified investigators in the field, it is also for sale by the Superintendent of Documents, Government Printing Office.

Project Title: Gastroenterology Abstracts and Citations

Contract Number: PH43-68-660

Amount: $90,730

Contractor: International Information Incorporated

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Dr. Kent Gershengorn
Dr. Michael Cohen

Man Years: Total: 1.60
Professional: .65
Others: .95

Project Description:

In January 1966 the NIAMD began publication of a current-awareness journal for gastroenterologists. Gastroenterology Abstracts and Citations appears monthly; each issue contains approximately 350 abstracts of the most significant gastroenterological literature published in the world and approximately 750 citations covering other relevant gastroenterological literature. Each issue contains a subject and author index. Cumulated subject and author indexes will appear at the end of the year. This new current-awareness publication was initiated to aid in the more prompt communication and dissemination of current research efforts in gastroenterology. Hopefully, it will also provide additional stimulation in the important research being carried out in the field of gastroenterology. Gastroenterology Abstracts and Citations is available, free upon request, to qualified investigators and practitioners in the field of gastroenterology as well as other government agencies with responsibilities in this area, medical schools, and hospital libraries. In addition to the free distribution of this publication to qualified investigators in the field, it is also for sale by the Superintendent of Documents, Government Printing Office.

Project Title: Artificial Kidney Bibliography

Professional Personnel: Dr. Keatha K. Krueger
Dr. Roger Lieberman

Man Years: Total: .8
Professional: .56
Other: .24

Project Description:

A recurring bibliography on kidney failure and artificial kidney has been developed through the MEDLARS program of the NLM and the staff of the Institute's Artificial Kidney-Chronic Uremia Program. Four quarterly experimental issues were prepared covering 1967 literature. These publications were distributed to the interested membership of the American Society for Artificial Internal Organs and individuals closely concerned with the Institute's artificial kidney program in order to explore utility, optimal content, and format. This publication was initiated on a regular quarterly basis in 1967 and covers the aspects of chronic kidney failure and artificial kidney available in the National Library of Medicine's literature collection.

The need for expansion of the coverage of this publication to also include engineering and physical and organic chemistry aspects of artificial kidney research has been evidenced, and plans are under way to implement this expansion. Abstracts of the most relevant (to the Artificial Kidney-Chronic Uremia Program) literature will also be initiated.

Project Title: Endocrinology Index

Contract Number: PH43-66-480

Amount: $45,265
(March 1966 thru March 69)

Contractor: Vanderbilt University (Dr. Grant Liddle, Principal Investigator)

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Dr. Roger Lieberman

Man Years: Total: .45
Professional: .35
Others: .1

Project Description:

Beginning with the literature processed by the National Library of Medicine in their Medical Literature Analysis and Retrieval System (MEDLARS) for 1968, the Endocrinology Index will be published bimonthly by the NIAMD. The citations included in this publication will be chosen automatically from the total literature included in Index Medicus and these citations, arranged according to subject categories, will constitute the first section of the Index. It will also contain a section of reviews which correspond to the same time interval. A numbered author section, which includes the index terms applied to each citation is a reference point for the author and subject index sections which complete the issue.

This publication is being produced and distributed on an experimental basis, pending Bureau of the Budget approval as a regular publication of the NIAMD.

Project Title: Nutritional Requirements of Vertebrates Other Than Man

Contract Number: PH43-64-44, Task Order No. 4

Amount: $82,000 (November 1963 - December 1968)

Contractor: National Academy of Sciences

Project Officer: Dr. Keatha K. Krueger

Man Years: Total: .1
Professional: .05
Others: .05

Project Description:

Work performed under this contract is a continuation of the studies of factors involved in assessing the nutritional requirements of vertebrates other than man. The project operates through an overall planning committee which selects species to be studied, need for revision based on the magnitude and significance of the new information available, and through the membership of subcommittees which are the work groups of the activity. The members of the committee receive no remuneration, but donate their time because of their own interest in the work of the committee and because they consider the final result to be of sufficient value to justify their efforts. The contract money is used to service the committee in terms of travel expenses, and secretarial and editorial services. The publications resulting from this work are considered standard reference materials for research workers and teachers in the field of animal nutrition. Since periodical revision is necessary to evaluate and incorporate the results of the continuing flow of new research, each year five to ten publications are in some stage of revision or preparation process.

Project: Publication of Proceedings of the Deuel Conferences

Contract Number: Not yet available.

Amount: $5,700

Professional Personnel: Dr. Keatha K. Krueger
Mrs. Ethaline Cortelyou

Man Years: Total: 0.09
Professional: 0.07
Others: 0.02

Project Description:

The proceedings of the 1967 conference on The Fate of the Dietary Lipids was published in February 1968. The 1968 conference on The Turnover of Lipids and Lipoproteins was held February 21-24, 1968. The proceedings will again be published by NIAMD. Camera-ready copy for the proceedings for printing and distribution through the GPO are being prepared by a personal service contract under the supervision of Dr. Laurance Kinsell and Dr. George Cowgill.

Project Title: Workshop on Epidemiological Studies on Osteoporosis

Professional Personnel: Mrs. Ethaline Cortelyou

Man Years: Total: 0.08
Professional: 0.06
Other: 0.02

Project Description:

A small working meeting of ten men was held October 23-24, 1967, to discuss progress in epidemiological studies on osteoporosis initiated in 1965 as a result of a WHO survey. Latest surveys indicate racial incidence, that it is greatest among fair people (especially redheaded women) of northwestern Europe and practically nonexistent among Negroes. Methods of measuring and comparing bone density were discussed. It is definitely age-related, particularly postmenopausal among women, but the dietary relationship is not as well established. Plans were made for establishment of study centers and collection and comparison of data. To accomplish this a protocol for conducting the studies is being prepared.

Project Title: Conference on Progress in Methods of Bone Mineral Measurement

Professional Personnel: Dr. Keatha K. Krueger
Mrs. Ethaline Cortelyou

Man Years: Total: 0.2
Professional: 0.1
Others: 0.1

Project Description:

This conference was held at NIH on February 15-17, 1968 with Dr. G. Donald Whedon, Director, NIAMD, as one of the co-chairmen and organizers of the conference. The purpose of the conference was to evaluate and compare techniques under development to relate bone density and mineral content to the degree of pathology in bone disease and to establish normal ranges of mineral content according to sex and age. Proceedings of the conference will be published in the Journal of Clinical Orthopaedics and Related Research in the early fall.

Project Title: Conference on the Nutritional Aspects of Uremia

Professional Personnel: Dr. John Magill
Dr. Keatha K. Krueger
Mrs. Ethaline Cortelyou

Co-sponsoring Agency: National Center for Chronic Disease Control

Man Years: Total: 0.25
Professional: 0.23
Others: 0.02

Project Description:

This working conference was held October 2-3, 1967 for the analysis and exchange of ideas on the current knowledge of dietary therapy in the management of patients with chronic renal failure. Special attention was focused on comparative evaluation of new types of diets and new dietary products, the use of which has permitted limited extension of survival and rehabilitation in certain types of patients with chronic renal failure. Many times the use of these dietary regimes with limited dietary nitrogen intake, including minimal amounts of the essential amino acids, have prolonged times between dialysis or made dialysis unnecessary.

The proceedings of the conference are being published as a special supplement to the American Journal of Clinical Nutrition. This was the first of a series of annual conferences, each to be co-sponsored by the NCCDC, devoted to some problem directly related to chronic renal failure and the Artificial Kidney-Chronic Uremia Program of the NIAMD.

Project Title: First Annual Contractors' Conference: Artificial Kidney Program

Professional Personnel: Dr. Allen Heim
Dr. Roger Lieberman
Dr. John Magill
Dr. Keatha K. Krueger
Mrs. Ethaline Cortelyou

Man Years: Total: 0.3
Professional: 0.15
Others: 0.15

Project Description:

This conference, held on January 23-24, 1968, brought together about 130 Artificial Kidney-Chronic Uremia Program contractors' key staff members with consultants to the Program and its staff. There was extensive review and study of existing contract-funded research and development projects, with emphasis on program progress and projected plans for the future. Proceedings of this conference will be available for distribution before the end of this fiscal year.

Project Title: Proceedings of a Conference on Natural and Synthetic Membranes

Professional Personnel: Dr. Kent Gershengorn
Dr. Keatha K. Krueger
Mrs. Ethaline Cortelyou

Man Years: Total: 0.04
Professional: 0.02
Others: 0.02

Project Description:

This conference on natural and synthetic membranes was the 21st Annual Conference of the Blood Research Institute and was held in Boston, November 8-9, 1967. Only the publication of proceedings is being supported by NIAMD.

Project Title: Guide to Nutrition Terminology

Contract Number: PH43-67-1449

Amount: $42,505 (50% paid by NIAMD)
(June 29, 1967 through June 28, 1968)

Contractor: Vanderbilt University (Dr. E. Neige Todhunter
Principal Investigator)

Project Officer: Dr. Keatha K. Krueger

Co-sponsoring Agency: NICHD

Man Years: Total: 0.45
Professional: 0.35
Other: 0.1

Project Description:

This project was initiated after considerable discussion with leading nutritionists regarding their needs in the information area. Representatives of the American Institute of Nutrition expressed particular concern. The major effort of the contract (co-sponsored equally with NICHD) is directed toward the development of a thesaurus of nutrition terms both hierarchical and alphabetical with their equivalent terms as used by organizations such as the National Library of Medicine and Chemical Abstracts Service. Definitions also will be included where needed. The thesaurus will serve as a guide to the definition of the science of nutrition; this is an important facet of the work since nutrition science tends to overlap a large number of other disciplines. Because of this overlap, there is no one information source for the nutritionist to consult for all bibliographic information in his field. This guide will aid him in asking for literature searches from several existing information sources. The guide will be published when completed.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

ARTIFICIAL KIDNEY PROGRAM

Summary Report

Background

During the last five years, diseases of the kidney, and especially end-stage kidney disease, have come to the forefront of attention for both Federal and non-Federal research and service efforts. Much of the impetus for this increasing interest in kidney diseases has come from the development of two dramatic yet expensive therapeutic approaches to end-stage disease - chronic hemodialysis (repeated treatment with an artificial kidney machine) and kidney transplantation.

The magnitude of the end-stage kidney disease problem, made increasingly apparent by the dramatic advent of artificial kidney therapy, is underscored by the latest available estimates from the National Center for Health Statistics of the Public Health Service indicating that about 50,000 Americans die each year of irreversible kidney failure. Of these, only from 6,500 to about 9,000 may be currently considered suitable candidates for the two modes of treatment mentioned above.

The problem of perpetual maintenance of patients with artificial kidneys is complicated by the fact that the cost of currently available techniques is too high, both in terms of money and qualified manpower, to permit widespread patient treatment. Also, patients maintained with the aid of artificial kidneys continue to display a number of undesirable symptoms related either to the underlying disease or to the treatment.

The Institute's special Artificial Kidney Program has been created to deal with these problems.

General Outline of the Program

The Institute's Artificial Kidney Program is an integrated research and development program aimed at the development of improved and more effective dialysis hardware and methodologies and of methods for optimal rehabilitation of chronic dialysis patients. The major portion of these activities is carried out through the contract mechanism.

The Program is centrally directed, and its research and development projects have been stimulated, directly and indirectly, by staff initiative. In the management, planning and analysis of this Program, the staff is strongly supported by 23 consultants in the field of kidney disease and hemodialysis. Among them are physicians who have led programs in artificial kidney centers, renal physiologists, physical chemists and membrane specialists, chemical and electronic engineers, and certain talented individuals who combine several of the above qualifications. These consultants are assisting in setting the trend of the Program and review the scientific merit, feasibility, and program relevance of contract proposals.

Research and development contract applications are subject to a double review. The first review, primarily for scientific merit and program relevance, is conducted by two alternating groups of about ten outside consultants each, who assign priority recommendations to each proposal. The final review is by a group made up of the Institute Director's staff (Institute Contract Review Committee) reinforced by four senior outside consultants, authorities in renal disease, hemodialysis and chemical engineering.

Program Content

The currently active projects constitute a broad spectrum of approach to well-identified problems of highest priority in this field. These projects involve development of improved blood cannulas and filtration membranes; evaluation and development of new polymer surfaces for artificial kidneys, cannulas and membranes which do not induce blood coagulation; design of more efficient, compact, and less costly dialyzer systems; development of a new concept of artificial kidney dialysis ("hollow fiber" or "capillary" kidneys); research on and development of absorption cartridges for blood purification; research on the utilization of ultrasonics to speed up dialysis in artificial kidneys; development of inexpensive disposable dialysis units; improvement and design of new automated centralized artificial kidney systems; design and development of prototypes for miniaturized or portable artificial kidneys; new approaches to artificial kidney methodology such as diafiltration (a novel concept in artificial kidney design); evaluation of special protein-restricted diets in the maintenance of uremic patients for whom chronic dialysis is deemed not feasible or unobtainable; isolation from dialysates and identification of the as yet unknown compound(s) which is (are) specifically responsible for the toxic symptomatology in uremia, and many other projects related to improved dialysis methodology or apparatus.

Main Subdivisions of the Program

Practically all existing research and development contracts can be arrayed among the following four main subdivisions:

(1) Membranes and mass transfer

(2) Hardware and instrumentation

(3) Blood cannulas and biologically compatible materials

(4) Toxic factors in uremia and dietary management of chronic uremia.

A fifth subdivision comprises a contract for the establishment of a long-term patient registry and medical information system to cover all patients maintained by chronic dialysis in the United States.

Accomplishments

A compact, high-efficiency dialyzer for artificial kidney machines has been developed which permits treatment of almost twice as many patients in a given dialysis center setting as with conventionally used dialyzers. The Babb-Grimsrud (B-G) dialyzer, named for its developers, measures only 20 by 50 centimeters and is constructed of plastic and a commercially available nickel foam support material. It requires a total blood volume of only 70 cc, thus decreasing the presently high blood priming requirement and the need for compensatory blood transfusions. It costs half as much as the conventionally used two-layer Kiil dialyzer, can be assembled in ten minutes and can be sterilized in an autoclave. Compared to the Kiil dialyzer, the B-G dialyzer has approximately one-third the membrane area, one-seventh the blood volume, and one-fourth the weight, with no decrease in urea clearance and only slight decreases in creatinine clearance. At the present time a series of these new dialyzers is being built for extensive testing under routine operating conditions.

The same clinicians involved in collaborating with the engineers on the Babb-Grimsrud dialyzer design at the University of Washington, Seattle, have devised a technique for storing and re-using artificial kidneys of the Kiil type and of the attached blood tubing, which reduces the cost and simplifies the work involved in performing hemodialysis in the home. The new procedure permits re-use of the artificial kidneys for at least three successive dialyses, requires assembling and disassembling of the unit only once instead of three times a week, and allows use of blood tubing sets at least nine times, for an estimated saving of $900.00 per year.

The new and unique hollow fiber capillary kidney, which is being developed under a contract with the Dow Chemical Company, is progressing according to schedule. Dialyses of dogs have been successful, and for the last few months this dialyzer has been used routinely in selected patients in northern California in a large scale clinical trial. The advantage of this design is that it is compact, disposable, and as efficient as dialyzers in current use, and lends itself to mass production. The design consists of small bundles of many hollow cellulosic fibers through which the blood is passed, and which are washed by a blood-purifying dialyzing solution. In addition to the above-mentioned two new artificial kidney designs, other possible designs are being explored including two other approaches to the hollow fiber dialyzer design.

Another contractor who is manufacturing the conventional Kiil dialyzer has been successful in the development of a new type of multiple, minute pyramidal membrane supports which increase the effective dialysis area of the membrane in the Kiil dialyzer. Preliminary laboratory tests have shown that this new development has increased the dialyzing efficiency of these modified Kiil dialyzers by about 40 percent. A series of the new type of dialyzer is now being built for clinical testing with patients.

The Program is believed to have had and will have other indirect salutary effects. The sudden vigorous activities in artificial kidney development (and the possibility of the new low-cost disposable capillary dialyzers which are being developed under Program contracts) are expected to exert a gradual effect on the price of commercially produced dialysis equipment. Such an effect has already been visible in the current price of the only commercially available disposable dialyzing cartridge. In the long run such developments are likely to result in distinct economies in dialysis treatment, particularly in home dialysis for which disposable types of dialyzers are well suited.

The activities of the Program have also resulted in an increase, in this country, in the use of a new type of diet, developed in Italy and England and heretofore used primarily abroad, which has permitted extended survival and definite rehabilitation in certain types of patients with chronic renal failure, where recourse to kidney transplantation or artificial kidneys is not possible or advisable.

Under the auspices of one contract a standardized, stable uremic monkey colony has been produced by graded, progressive nephrectomy. Such animals, not previously available, can now be used to study their reaction to isolated fractions of uremic plasma or dialysate presumed to represent specific toxic factors.

Under the auspices of another contract a detailed, comprehensive instruction manual has been produced for use by patients presently on chronic dialysis in hospital centers who are being shifted to maintenance with the aid of independent dialysis in the home--a less expensive method of maintenance which also comprises a number of other desirable features.

Publications

In 1967 the Program began publication of the quarterly ARTIFICIAL KIDNEY BIBLIOGRAPHY. This current-awareness publication provides information on the latest scientific articles published anywhere in the world concerning renal failure, dialysis, artificial kidneys, and kidney transplantation. It is made available, without cost, to NIH grantees engaged in kidney disease research, to contractors of the Artificial Kidney Program and, upon request, to other qualified interested investigators and practitioners working in areas closely related to the artificial kidney. Recipients of this new publication have indicated with enthusiasm its usefulness to research and to patient care.

The Program has also published and widely distributed the publication "A Review of the Development and Performance of Hemodialyzers" which was prepared by one of its contractors. Among other essential subject matter it contains tables which, for the first time, compile and compare performance data on the various existing dialyzers and dialysis membranes - an indispensable aid to anybody engaged in artificial kidney improvement.

Conferences

A working conference on the dietary management of chronic renal failure, co-sponsored by the Artificial Kidney Program of the Institute and the Kidney Disease Control Program, BDPEC, PHS, was held October 2-3, 1967. The two-day session was designed to analyze and communicate certain aspects of current knowledge of dietary therapy in the management of patients with chronic renal failure. Special emphasis was devoted to a comparative evaluation of a new type of diet, developed in Italy and England, which limits dietary nitrogen intake to minimal amounts of essential amino acids, and which has permitted limited survival and rehabilitation in certain types of patients with chronic renal failure, without recourse to kidney transplantation or artificial kidneys.

As a result of the meeting a number of pertinent clinical studies will be undertaken by several participants, in addition to the work already currently supported by the Program in this area, with special emphasis on the use of variations of this diet coupled with occasional, infrequent dialyses in the many patients who cannot now obtain regular chronic dialysis treatment. The proceedings of the Conference have been published as a special supplement to the monthly American Journal of Clinical Nutrition and copies will be made available to all workers in the field of chronic renal failure.

As a result of the success of this Conference a similar conference on abnormal calcium metabolism and bone disease in uremia and dialysis patients is planned for the fall of 1968.

During the past year an important series of conferences was initiated. They are planned to be yearly conferences for the contractors in the Artificial Kidney Program. The first annual Contractors' Conference was held on January 23-24, 1968, and brought together about 130 Artificial Kidney Program contractors' key staff members with consultants to the Program and its staff. There was extensive review and study of existing contract-funded research and development projects with emphasis on Program progress and plans for the future. Proceedings of this Conference are being published this summer.

Patient Registry

A patient registry coupled with a medical, engineering and economic analysis of all patients in chronic dialysis has been instituted with the Research Triangle Institute as the contractor. The contract provides for a registry of all patients presently maintained with the aid of artificial kidneys or peritoneal dialysis, and will permit a truly comparative evaluation of the various facets, steps and methodologies imvolved in chronic dialysis, from selection of the patient through his medical evaluation once he is being maintained with chronic dialysis. This overall systems analysis will be undertaken as a cooperative effort with the community service programs in dialysis of the Kidney Disease Control Program of the PHS; it will cover the various dialysis centers of the Veterans Administration Hospitals and all other centers or hospitals engaged in chronic dialysis and willing to cooperate.

In addition, there has already been an exchange of information with the personnel involved in the annual dialysis survey of the European Dialysis and Transplant Association concerning information obtained during its annual cross-section survey of the patient population in Europe, which equals approximately the number of patients on dialysis in this country. Since a number of valuable innovations has originated in Europe and since the patient problems are similar, access to the European data and a mutual exchange of information will increase the value of both registries, particularly as to the medical, clinical, and technical aspects of dialysis.

Future Directions

In the near future the Program will also place special emphasis on clinical studies which are intended to aid in the definition of the pathogenetic mechanisms of the serious accompanying features of renal failure during chronic dialysis. Three principal features have been identified: peripheral neuropathy, calcium dystrophy, and anemia; it is not clear whether these frequent clinical derangements are by-products of the imperfection of current dialysis methods or sequellae of long-continued uremia hitherto unobserved because of shorter life duration prior to development of dialysis treatment. Clinical studies intented to clarify the pathogenicity of these complications and to evolve ways and means of avoiding them are considered essential for a satisfactory degree of rehabilitation of patients maintained by chronic intermittent dialysis. Efforts are already underway to organize small selected collaborative clinical studies along these lines.

Summary

The Institute has implemented a direct, and planned program of contract-funded research and development aimed at obtaining, via the shortest possible route, a lowering of cost and improvements in operational efficiency of current methods of dialysis to provide for improved, permanent maintenance and rehabilitation of patients with chronic kidney failure. The following pages give a more detailed enumeration of the currently active research and development projects of this Program.

Contractor: Abcor, Incorporated

Amount: $115,713

Title: Development of a Capillary Membrane Dialyzer

Objectives:

To develop an improved artificial kidney using small diameter capillary membranes as its basic elements.

Major Findings:

Work has progressed along several lines towards the goal of developing a utilizable capillary membrane kidney. Means of obtaining hollow fibers with permeation properties in the range of existing membranes appears to have been achieved. Procedures for treating these fibers to yield urea diffusivities approximately equal to those of cellophane have been developed. Residence time distribution measurements in bundles of hollow fibers indicate that dispersion of flow within them is quite small, of the order of that due to normal laminar flow in a single duct. Basic design characteristics have been determined, but remain to be optimized.

Proposed Course:

With the attainment of well-controlled and reproducible capillary membranes, a complete unit will be fabricated. *In vitro* and *in vivo* tests will continue with the goal of initiating clinical testing.

Contractor: Aerojet-General Corporation

mount: $141,002

le: Improved Membranes for Hemodialysis

Objectives:
To develop improved membranes for hemodialysis with respect to permeability features and surface thrombogenicity

Major Findings:
Thus far, hydrolyzed and cellulose acetate membranes with enhanced permeability have been developed. Some of these membranes ultra-filter water at 30 - 40X the rate of the presently used cuprophone. A cellulose polymer containing a high quantity of amino groups for hepinarization has been prepared.

Contractor: Aerojet-General Corporation

Amount: $183,268

Title: Inexpensive Manufacturing Method for the Kiil Artificial Kidney

Objectives:

This is a new program directed at applying an electro-plating mold technique to the manufacture of Kiil boards. This method should result in a cost reduction of over 50% in the present Kiil unit.

Contractor: Albany Medical College

Amount: $25,700

Title: Electronic EEG Frequency Analysis for Evaluation of Uremia

Objectives:

To perfect a simple, inexpensive, practical EEG frequency analyzer which will permit wide usage of EEG frequency analysis in evaluation and study of uremia. The equipment necessary for the performance of this study has been carefully selected and the actual evaluation of EEG's in uremic patients is presently underway.

Contractor: Anatole J. Sipin Co.

Amount: $49,930

Title: Blood Mass Transfer Study Using Jet-Wave Principle

Objectives:

This is a new program designed to study the merits of the jet-wave principle relative to the overall mass transfer of undersirable components in the blood which accumulate in uremia. To accomplish this study the contractor will design, construct and *in vitro* test an experimental scaled model of a one turn circulating jet wave hemodialyzer in which the jet wave is produced by a rotating jet drum. The model will contain a membrane surface area of approximately 1/3 that of a twin coil artificial kidney. The model will be flexible in design to permit changes in configuration and variations in operating parameters. This flexibility will provide for such factors as, adjustment of width of jet slits so that various combinations of number of jets, jet pressure and dialysate flow rate can be examined.

The results of this study are intended to lead to the utilization of this principle for a full-scale hemodialyzer.

Contractor: Anatole J. Sipin Co.

Amount: $27,832

Title: Dialysis Blood Flowmeter

Objectives:

This contract was a feasibility study for the development of a dialysis blood flowmeter utilizing a flowmeter principle which has been used in large volume flowmeters. In this design, the cannula or connecting tube is vibrated transversely to the flow path and dynamic reactions of the vibration are sensed as a measure of mass of rate of blood flow through the tube.

Major Findings:

During the first eight months of this contract the feasibility of adapting this principle to low flows has not been demonstrated. With low flows, resistance to vibration caused by the cannula tubing is greater than that caused by the mass of blood flowing through the tubing. New, more flexible materials have not been found to circumvent this problem. Furthermore, a flowmeter utilizing the Doppler principle has since become commercially available. The adaptability of this Doppler flowmeter to dialysis cannula flow measurement has reduced the priority for further research into this area.

Contractor: Battelle Memorial Institute

Amount: $64,705

Title: Development of Improved Hemodialysis Membranes

Objectives:

Development of nonthrombogenic, high strength, high permeability membranes for artificial kidney use.

Major Findings:

Three approaches have been followed in the development of membranes for use in artificial kidneys: 1) crosslinking of water-soluble polymers, 2) membrane preparation from water swellable but insoluble polymers, and 3) transformation of essentially non-swellable polymers. Best results have been obtained by casting nylon-based membranes from organic solvents including controlled amounts of dimethylsulfoxide. The addition of a modifying resin has led to products of greater permeability and durability. The best of the modifiers examined so far are epoxies and sulfonamides. The method of membrane preparation has been found to exert appreciable influence on the dialysis properties.

Proposed Course:

Preparative factors which influence permeability-strength relationships of membranes, and the preparation of nonthrombogenic surfaces will be investigated with continuing emphasis on the nylon-epoxy membranes.

Contractor: Battelle Memorial Institute

Amount: $98,100

Title: Development of an Improved Artificial Kidney System

Objectives:

To demonstrate the feasibility of hemodialysis utilizing micro-encapsulated absorbtion reagents.

Proposed Course:

Various chemicals will be tested for their ability to bind urea. These reagents will be surrounded by a permeable polymeric material which will allow water and urea (as well as other compounds) to diffuse into the microcapsule where the latter will be bound. The coating material will not allow the binding material to diffuse out.

Contractor: Becton, Dickinson and Company

Amount: $60,678

Title: Hemodialysis Cannulization Development

Objectives:

Development of improved hemodialysis cannulae in order to minimize or overcome the problems of clotting and infection in the long-term care of chronic renal failure patients. This objective will be pursued in the following areas: (1) Improvements of the coupling to the external hemodialysis shunt and (2) the development of an improved hemodialysis venous cannula for the internal arterio-venous fistula shunt technique.

Contractor: Blood Research Institute (Formerly Protein Foundation)

Amount: $33,800

Title: Determination of Membrane Characteristics of Polyionic and Other Polymeric Membranes

Objectives:

To investigate the characteristics of a wide variety of membranes as to their capabilities as the partitioning structure in dialysis and ultra-filtration. The project includes the study of protein retention characteristics of the membranes and the testing of protein solutes for any physical, chemical or biological changes.

Major Findings:

Several tentative conclusions can be drawn from the studies performed: (1) when serum proteins contact the amicon PM-30 and UM-1 membranes, the membrane characteristics are irreversibly changed; (2) once serum protein polarization occurs, the passage of microsolutes is not passive, but is directed by the protein in the membrane, while fluid transfer is decreased; and (3) the influence of pressure on these membranes within the pressure range of 90 mm Hg to 50 pounds per square inch is significant only in the absence of serum globular proteins. Once membrane caking occurs, pressure has little influence on fluid transfer.

During *in vivo* experiments, the membranes studied to date do not cause denaturation of serum proteins.

Proposed Course:

To continue evaluation of membranes for use in artificial kidneys under various pressures and with standardized protein solutions and microsolutes.

To study the interaction of the plasma proteins of patients with the membranes and other components of artidicial kidneys under various pressures and with standardized protein solutions and microsolutes.
To study the interaction of the plasma proteins of patients with the membranes and other components of artificial kidneys including observing enzymatic and immuno globulin changes, and the adsorption of plasma proteins to the membranes.

Contractor: Case Western Reserve University

Amount: $36,663

Title: Removal of Waste Metabolities from Dialyzing Fluid by Microencapsulated Reactants

Objectives:

To develop a new technique for removing waste metabolities from dialyzing fluid by encapsultating specific reactants inside hollow polymeric spheres.

Major Findings:

Satisfactory progress has been made in both microencapsulation technique and conversion of Dowex A-1 to the cobalt form. A technique has been developed in preliminary form for the production of relatively uniform microcapsules by interfacial polymerization of nylon from hexamethylenediamine and sebacoyl chloride. The capacity of Dowex A-1 for divalent cobalt has been measured, and preliminary information has been obtained on the stability of the resin-cobalt complex, oxidation of the cobalt to the trivalent state, and removal of ammonia from aqueous solution by the resin in the cobalt form.

Proposed Course:

Work is proposed to continue the development of microcapsules for the removal of metabolites from dialyzing fluid. Attention is to be focused on: (1) microencapsulation techniques, membrane characterization and control of mass transfer characteristics; (2) means of removal of urea through hydrolysis and CO+++ complexation of the resulting ammonia or direct reaction in a polyacid environment within a microcapsule (xanthydrol or Micklers Ketone).

Contractor: Case Western Reserve University

Amount: $40,855

Title: A Research Program Leading to an Improved Artificial Kidney

Objectives:

1. To develop highly permeable membranes.
2. Increase mass transfer through laminar layer at membrane surfaces.
3. Develop a new technique for measuring very small motions of fluid in a sensing volume of only a few microns.
4. Develop an improved dialyzing unit design through use of an optical heterodyne spectroscope.

Major Findings:

1. Membranes
    - a) Techniques and apparatus have been developed to permit (1) reproducible casting of modified cellulose acetate membranes in a controlled atmosphere, and (2) determination of permeabilities of the membrane to water, small solutes and blood proteins.
    - b) Casting techniques have been developed permitting the fabrication of membranes having water permeabilities up to 50-fold higher than that of typical cellophane dialysis tubing, while exhibiting better retention of bovine serum albumin.
    - c) Membranes have been reinforced with nylon monofilament cloth resulting in membranes having wet tensile strength 2.5 times higher than that of cellophane, without appreciable sacrifice in water permeability.
    - d) Initial work has been done on the study of membrane structure, aiming at the correlation of permeabilities with casting variables and membrane morphology.
2. Laser Doppler Flowmeter
    - a) Work has been done on a laser Doppler flowmeter which has resulted in the completion of construction of the flow system, test chambers, optical systems and electronic detection equipment. The apparatus has been successfully used to detect heterodyning between various transverse laser modes.

      Velocites as slow as .005 centimeters per second (0.6 feet per hour) have been measured. The device provides a method of measurement not possible in any other way. This instrument will be used in obtaining data to aid in optimizing the design of artificial kidneys. It is expected that the effects of membrane and support configurations on the flow regime and mass transfer near the membrane will be accurately determined, so that optimization can result. The ultimate spatial resolution is limited by diffraction effects which become important when observing volume elements approximately 5 microns in diameter.

It is expected that this instrument will measure relative changes in refractive index of one part in 10^8. Therefore, concentration profiles and changes can be measured much closer to the membrane and with greater precision than is possible with any other instrument. It is estimated that the concentration can be examined to within about 5 microns of the membrane surface.

Proposed Course:

1. To continue the development of highly permeable membranes.
2. To continue the development of an improved dialyzing unit design through the use of an optical heterodyne spectroscope.

Contractor: Cedars-Sinai Medical Center

Amount: $213,682

Title: Divalent Ion Metabolism and Renal Osteodystrophy in Chronic Renal Failure and Effect of Chronic Hemodialysis and Renal Transplantation

Objectives:

The objective is to evaluate the mechanisms leading to deranged divalent ion metabolism in chronic renal failure and to determine rational modes of therapy to correct it. Various tests on uremic patients will include calcium, magnesium, and phosphate clearances; hydroxyproline and pyrophosphate clearance, parathormone and Vitamin D assays; calcium absorption and infusion tests; parathormone infusion tests and Vitamin D administration response.

Contractor: Columbia University

Amount: $156,493

Title: Identification of the Non-Thrombogenic Environment

Objectives:

The goal is to identify those characteristics of surfaces and blood which may make an atraumatic, non-thrombogenic environment possible. An intravascular test device has been designed to permit the *in vivo* testing of artificial materials under controlled velocities. Critical parts of the device will be removed from contact with possibly damaged intima and released tissue thromboplastin. The role of various surfaces, turbulent flow, platetlets, coagulation factors, temperature, and blood velocity on the initiation of clotting will be investigated. Various histologic, chemical, and physical manifestations of clotting will be measured.

Contractor: Columbia University

Amount: $39,639

Title: Specific Extra-Corporeal Absorbents

Objectives:

This is a new program designed to synthesize and test various cationic, anionic and non-ionic polymeric absorbents for their ability to remove undesirable components which accumulate in uremia. After suitable materials have been prepared, it is intended to propose a joint study with renal physiologists and clinicians to use these materials to isolate and identify other toxic agents and to test those materials *in vivo* employing animals.

Contractor: Columbia University

Amount: $97,347

Title: Development of a Low Cost Artificial Kidney

Objectives:
The objective is to develop an optimized prototype of a tidal-flow artificial kidney.

Major Findings:
Although a prototype disposable kidney is not ready for clinical testing several technical achievements have resulted from the investigation: (1) a multiplex arrangement of units has been formed into a single cartridge configuration; (2) new frame materials (closed-cell foamed plastics) were evaluated and new assembly methods were developed; and (3) a membrane treatment was developed which permits dry assembly of cartridges without subsequent dimensional alterations when wetted immediately prior to use.

Proposed Course:
Design and materials studies are proposed to permit the development of a low cost, effective, functional unit.

Contractor: Columbia University

Amount: $13,711

Title: Standard Test Methods for Evaluation of Membranes

Objectives:

A standard membrane test cell will be developed that can give significant hydraulic permeabilities and ultrafiltration indices of various membranes. It will employ turbulence-producing spacers adjacent to the membrane face, which will reduce unstirred film resistance without producing membrane flutter. An ultrafiltration test cell device will also be built, tested, and modified as necessary.

Contractor: Dow Chemical Company

Amount: $294,100

Title: Research on Hollow Fiber Dialyzer Units

Objectives:

To develop a hollow fiber dialyzer as an efficient, low cost kidney.

Major Findings:

During the past contract year, a miniplant was designed and constructed with a potential production capacity of 60 kidneys per 8 hour shift, and which should be adequate for providing continuing research requirements. Provisions have been incorporated into the design for sterilization, packaging, and storing of the kidney in a dry state or in a wet state with the fibers filled with a sterile solution. This should provide the patient with a hollow fiber kidney which can be prepared with a minimum of effort for hemodialysis.

The first clinical trials utilizing the hollow fiber kidney on humans were conducted. At Marquette University, 23 dialyses of chronically uremic humans established the feasibility of the device for hemodialysis. No untoward reactions of the patients were noted. In addition, at San Francisco General Hospital approximately 100 dialyses have been performed in the initial stages of more exhaustive clinical trials. Urea, phosphate and creatinine clearances *in vivo* appear to be better than with the Kiil. These clinical trials are continuing.

Proposed Course:

Continuing clinical trials will provide baseline information concerning the kidney design and its clinical application. Further investigation of manufacturing-processing parameters, the investigation of improved permeabilities and of nonthrombogenic components of the complete system, should lead to the attainment of the long-term goals of the program.

Contractor: The Epoxylite Corporation

Amount: $196,048

Title: Development of Permanently Retained Percutaneous Implant Conduits and Safety Connector for Hemodialysis

Objectives:

To develop ancillary equipment for hemodialysis devices, with particular regard to the percutaneous shunt and connectors. Studies will be performed toward the development of (1) a fluid-proof, bacterial-proof percutaneous conduit, intended to enhance the performance of the Scriber shunt by markedly reducing infection in chronic dialysis patients, and (2) a positive-locking safety connector, intended to improve ease with which a patient may connect himself or be connected to an artificial kidney and to improve the over-all safety and integrity of the Scriber shunt.

Contractor: F M C

Amount: $186,000

Title: Development of an Improved Artificial Kidney Machine and Membrane

Objectives:

To produce an improved artifical kidney and membrane to be used in hospitals and in the home for the treatment of chronic renal insufficiency. The objectives of the design will be minimum cost and disposability, compact size, ease of operation, fail-safe monitoring and minimum set up and cleaning time. The initial design concept involves the use of vertical jets of fluid impinging on a membrane envelope in order to more efficiently propel the blood and scrub the membrane.

Contractor: Franklin Institute Research Laboratories

Amount: $20,000

Title: Nonthrombegenic Surfaces prepared by Glow Discharge Polymerization

Objectives:
Study the feasibility of using the technique of Glow Discharge Polymerization to produce negatively charged surfaces that remain negative for at least six months and have significant antithrombogenic activity as indicted by a prolonged Lee White clotting time.

Contractor: General Electric

Amount: $166,425

Title: Development of a Low Cost Artificial Kidney and Dialysis System

Objectives:
To develop a disposable, low cost, pre-sterilized, light weight artificial kidney and dialysis system - primarily for use in the home.

Major Findings:
This is a newly activated program. Preliminary work has indicated that Lexan or polypropylene are the materials of choice. Early hydrodynamic studies have indicted that a short flow-path length is desirable as compared to long-paths where resistance to blood flow may be excessive. Consequently early work suggests that repeating basic units either in a series-parallel arrangement or a stack assembly will provide an effective unit.

Contractor: Georgetown University

Amount: $99,724

Title: New Approaches to the Design of Chronic Dialysis Cannulae
for the Prevention of Infection and Clotting

Objectives:

The objective is to utilize materials which retard the clotting of blood in the fabrication of new arteriovenous cannulae for chronic dialysis patients. Hepacone, a mixture of heparin and silicone rubber, is one such material which has shown promise as a non clotting surface in initial animal cannula trials.

New designs of cannulae are also being developed to prevent infection at the site of the cannulae's exit through the skin. Materials are being investigated which will promote tissue ingrowth. A collar made from these materials is implanted subcutaneously to obtain tissue fixation - infection barrier. Other techniques, such as incorporation of antibiotics into the cannulae and skin sealing materials are also being considered.

Contractor: Gulf South Research Institute

Amount: $58,157

Title: A study of Synthethic Polypeptides as Possible Hemodialysis Membranes

Objectives:

To polymerize commercially available amino acids, form the product into membranes and test the resultant film for their potential usefulness as hemodialysis membranes.

Contractor: Hamilton Standard, Inc.

Amount: $95,000

Title: Automatic Peritoneal Dialysis Machine

Objectives:
To design, fabricate, and test a machine for intermittent peritoneal dialysis.

Major Findings:
An automatic peritoneal dialysis machine has been designed and constructed. The machine will deliver and withdraw equal volumes from the peritoneal cavity over a variable range determined by the physician. For example, infusion volumes can be 500-1500 cc deliverable over 5-20 minutes. The dialysate solution ratio can be varied from 25:1 - 35:1. Alarms will provide warning of pressure, temperature, and conductivity problems if they arise.

Proposed Course:
Clinical trials are planned to provide basic information on day-to-day machine performance. Subsequent changes will be made as indicated as a result of these trials.

Contractor: Hamilton Standard

Amount: $124,700

Title: Site Preparation Studies for a Peritoneal Dialysis Access Plug

Objectives:

To study the feasibility of creating a permanent access site or access button site in the abdominal wall of an animal to accommodate a through-skin connector for peritoneal dialysis.

Major Findings:

Four site preparation concepts, designed for easy and frequent insertion of peritoneal catheters for use in chronic peritoneal dialysis, have been evaluated. None of the preparations investigated, which include the simple stab wound, direct anastomosis of pertioneum to skin, velour surfaced insertion plug, and intestinal fistula have been successful in providing permanent access.

Proposed Course:

To continue with the intestinal fistula and velour ingrowth concepts in an attempt to alleviate the problems already encountered with these preparations. Species differences and bacteriological and histological changes will be examined.

Contractor: Harris Research Laboratories

Amount: $62,250

Title: Improvement in Cannulas for Hemodialysis

Objectives:

To improve cannulas for hemodialysis by designing and producing improved cannula tips, preparing new materials for anticlotting surfaces inside the cannulas and designing and producing improved means of mechanically anchoring the cannulas with a protective collar under the skin to act as a barrier against infection.

Major Findings:

Most of the progress was made in the development of soft tipped cannulas. A number of designs were made with the evolution of two basic types: 1) A very soft, cylindrical tip made of General Electric RTV 615 rubber reinforced with knit, woven or nonwoven Dacron fabric, and 2) tapered cylindrical tips made of a series of industrial grade silicone rubber.

A limited amount of experimental _in vivo_ testing in dogs was done.

Proposed Course:

This contract was allowed to terminate because of the inability to get sufficiently active vascular surgical collaboration and involvement on the project.

The fabrication and design techniques, however, may be of value to other groups working toward developing improved cannulas.

Contractor: Harvard University

Amount: $83,850

Title: The Formation of a Portable Artifical Kidney

Objectives:

To develop a compact artifical kidney through the use of ultrafiltration or other selective membranes. This includes the determination of selective filtration characteristics of ultrafiltration membranes and the *in vivo* study of clearance rates in normal and uremic animals.

Major Findings:

There has been further development of an *in vivo* circulatory whole blood system for the biological evaluation of the artifical and natural membrane materials which are intended for use in an artifical kidney device. Continuous monitoring techniques were developed for following the blood levels of urea, uric acid, glucose, sucrose, inulin, C^{14} urea, S^{35} sulfate and H^3 water. A dual-membrane comparative hemodialysis unit of similar membrane surface areas, channel widths, depths, and volumes was designed and the prototype constructed. Extracellular fluid space and body compartmental volumes have been measured in the rat.

Contractor: Hittman Associates, Inc.

Amount: $51,843

Title: An Investigation of Fluid Dynamic Techniques for the Improvement of Dialysis Efficiency

Objectives:

This is a new program designed to investigate the fluid dynamics of dialysis channels on the blood side to improve mass transport. The investigator will focus his efforts on determining the relative effects of perturbing the blood flow on the efficiency of dialysis and on the damage to blood constituents as a result of this perturbation. Perturbation will be accomplished either by hydrodynamic design of the blood channel or by mechanical agitation.

Based upon these fluid dynamic studies, experimental models of dialysis channels will be designed and constructed to test the design principles.

Contractor: Illinois Institute of Technology Research Institute

Amount: $74,267

Title: Optimized Cellulose Membranes for Artificial Kidney Dialysis Applications

Objectives:
The objective is to define cellulose membranes designed for use in hemodialysis and elucidate the fabrication and processing parameters which will enable them to be produced reliably and reproducibly.

Major Findings:
Cellulose membranes have been prepared using a procedure which is comparable to that used to prepare commercial Cuprophane. Several modifications of the membrane have been carried out in order to investigate the factors which most affect characteristics of strength and permeability.

Proposed Course:
Further efforts will be directed toward reproducibly obtaining improved membranes through modification of temperature of casting solution, monaxial and biaxial stretching, and inclusion of plasticizers into membrane framework. Membranes will be produced which are large enough for use on a standard Kiil dialyzer.

Contractor: Illinois Institute of Technology Research Institute

Amount: $76,726

Title: Feasibility Study on the Use of Ultransonic Energy to Increase Hemodialysis Rates

Objectives:

To test the feasibility of enhancing dialysis rate by application of ultransonic energy to dialysis membranes under varying conditions. The membranes are fixed in a special cell or are part of a regular-sized artificial kidney unit.

Major Findings:

Early in the study it was shown that a 3-5 fold increase in urea dialysance in a small cell and a 17% increase in sodium chloride dialysance in a large Travenol artificial kidney could be obtained. The dynamics of the system were studied in a small lucite constant flow cell rather extensively. The membrane in this system is supported by a mylar screen. It was found that the observed increases in crystalloid flux were due to local "hot points" or temperature elevation despite utilization of a constant temperature bath.

With the removal of the mylar window it has been possible to reproducibly demonstrate a 300% increase in Nacl dialysance with ultrasound. It appears that the mylar window damped the acoustic effect and a standing wave had not been generated in its presence. This large increase was demonstrated at low flow rate and when done in the presence of blood there was no observable increase in hemolysis by routine hemotologic studies.

A variable dialysis channel is being constructed for further evaluation of the ultrasonic effect.

Proposed Course:

1. To continue a detailed analysis of the mechanism of ultrasonic enhancement in respect to particle interaction, particle size, and electrical properties of the species, as well as, membrane type.
2. To extend the studies of the ultrasonic effect on formed elements and blood protein.

Contractor: Illinois Institute of Technology Research Institite

Amount: $158,709

Title: Identification of Toxic Components in Uremia

Objectives:

Utilizing peritoneal dialysate from chronically uremic and normal individuals, the objective is to isolate potentially toxic compounds from the following groups: peptides, non peptide nitrogenous substances, biologic acids and guanido derivatives.

Major Findings:

Utilizing dialysate collected aseptically, an attempt was made to find behavioral tests that would discriminate between uremic and non uremic dialysate. None of the tests met this criterion and it was felt that this was due to the small quantities of active material in the dialysate relative to the high concentration of glucose and electrolytes. A potentially useful method for eliminating the normal constituents from the dialysate has been proposed.

Contractor: Illinois Institute of Technology Research Institute

Amount: $40,089

Title: Optimization of membrane support in the Artificial Kidney with Ultrafiltration

Objectives:

To survey available porous materials to determine which, if any, would be potentially good support materials.

Major Findings:

An experimental flat plate dialyzer was constructed with interchangeable supports. Fourteen different materials have been investigated. However, it is too early to determine whether any of these materials offers economic or operational advantages over the materials now in use. This information should be available at the end of the project year.

Contractor: Jefferson Medical College

Amount: $28,079

Title: The Isolation, Identification and Quantitative Evaluation of Lipochromes and Urochromes in Normal and Uremic Patients

Objectives:

An attempt to determine the type of pigments present in normal urine will be made. Isolation of these pigments from normal and uremic plasma will be attempted. A correlation of the level of pigment in plasma and dialysate with the clinical status of the patient will also be made.

Contractor: Marquardt Corporation

Amount: $185,956

Title: Absorbent for the NIH Chronic Uremia Program

Objectives:

To develop sorbent materials and techniques for removal of waste materials from body fluids via the gastrointestinal tract or from shunted blood or lymph. The materials so developed might also be used to improve the effectiveness of conventional techniques of dialysis.

Major Findings:

The inital work was directed toward an attempt to find a sorbent that would remove the urea molecule from the dialysate solution. A system was developed whereby urea in the dialysate is converted to ammonium carbonate utilizing the enzymatic action of urease and the ammonium ion is subsequently removed by zirconium phosphate.

Proposed Course:

Continued development of the zirconium phosphate sorbent system for urea will ensue. With the use of additional sorbent materials, studies will be conducted in order to develop a highly efficient sorbent system for the removal of other waste products which are extracted from the blood of uremic patients by means of a conventional artificial kidney system. Work will also continue toward the goal of encapsulting sorbent materials, thereby permitting internal application of a sorbent via the gastrointestional tract, the peritoneum, the rectum, or directly in the blood stream.

Contractor: Massachusetts Institute of Technology

Amount: $279,675

Title: Development of Artificial Kidneys having Noncoagulating Surfaces

Objectives:

To construct pilot models of artificial kidneys using exclusively nonthrombogenic materials in order to demonstrate the feasibility of dialysis on uremic patients without the addition of heparin to the patient's blood.

Major Findings:

During the two years of this project, work has been devoted toward making cellulose surfaces nonthrombogenic to blood by binding heparin to them. Satisfactory heparin binding has been demonstrated but problems of heparin leaching and platelet build-up have yet to be solved.

Engineering research leading to miniaturization of an artificial kidney is being carried out.

Improvements in a dialyzer test cell to allow determinations of the dialysis characteristics of new membranes have been accomplished, and testing of new membranes is under way.

Contractor: Mercy Hospital

Amount: $162,330

Title: Development of a Permanent Upper Extremity Arterivenous Shunt

Objectives:

The objective is to develop a permanent arteriovenous shunt for use in chronic intermittent hemodialysis utilizing an approach involving promotion of tissue cover of the prosthetic surface. This involves the implantation of small diameter prosthetic vessels made from extremely finely knitted Dacron fibers. Tissue ingrowth into the interstices of the knitted material provides a stable layer of permanent host tissue. This objective has been accomplished in large vessel prostheses. The developmental aspect of this program involves defining the parameter required for small diameter vessels and adapting the prosthesis to the rather specialized function of an arteriovenous shunt.

Contractor: Monsanto Research Corporation

Amount: $162,974

Title: Research and Development on 1) Nonthrombogenic Surfaces and 2) Collagen Dialysis Membranes

Objectives:

1. An attempt will be made to develop an ideally blood compatible surface by chemically coupling blood proteins to the surface of suitable polymers.
2. Evaluate the suitability of the collegen membrane as a potentially useful dialyzing film that improves solute transfer and is non antigenic.

Contractor: Monsanto Research Corporation

Amount: $241,541

Title: Research and Development in Material and Devices for the Artifical Kidney

Objectives:

1. Evaluate new materials for use as membranes in hemodialysis
2. Prepare low modulus cannula tips, with a tissue compatible lining, from blood inert polymers
3. Develop a prototype dialyzer composed of hollow fibers

Major Findings:

A number of methods of synthesizing membranes have been investigated. Polyvinyl alcohol films cross-linked by heat have shown promising dialysis properties and moderate strength. A prototype tubular dialyzer has been developed with nylon as the membrane and Flow characteristics of this unit are being evaluated.

Proposed Course:

Development of a nonthrombogenic membrane with dialysis and strength characteristics that are equal to, or better than, cellophane. Develop a hollow fiber kidney with nonthrombogenic enclosures and new fibers of a diameter and dialysance suitable for clinical use.

Nonthrombogenic, tissue compatible, cannulae will be developed.

Contractor: Montreal General Hospital

Amount: $18,500

Title: Determination of Nutritional Requirements and Study of Body Composition in Patients on Chronic Hemodialysis

Objectives:

To determine the nutritional requirements and to study the body composition of patients on chronic hemodialysis. Longitudinal studies involving these patients include diet surveys, nutritional status, and balance studies for calcium, magnesium, zinc, iron, fat, nitrogen, amino acids and potassium.

Major Findings:

Balance studies of calcium and magnesium indicate that dietary calcium supplementation is necessary and that a dialysate level of 1.5 mEq/L of magnesium should be maintained. Bone density studies reveal no significant change in metacarpal cortical thickness in 18 or 20 patients studied.

Proposed Course:

The specific aims of future research are to continue these longitudinal studies.

Contractor: Naples University School of Medicine

Amount: $43,000

Title: Research related to Artificial Kidney Development and Chronic Uremia

Objectives:

This is a newly instituted program to (a) investigate some derangements of amino acid metabolism in uremic patients and study the pathophysiologic implication of the alterations (b) study nutritional and mass transfer aspects of supplementation of dialysate with selected amino acids and (c) explore the possibility of increasing the dialysis interval in chronic uremics by treating the patients with a selected low protein diet.

Contractor: Naples School of Medicine

Amount: $23,005

Title: Research Related to Artificial Kidney Development and Chronic Uremia Studies

Objectives:

To evaluate the use of oxystarch, a urea trapping compound which is not absorbable from the gastrointestinal tract, as an orally administered agent to reduce the blood urea concentrations in patients with chronic renal failure.

Contractor: National Bureau of Standards

Amount: $75,000

Title: Standard Test Cell

Objectives:

To prepare a membrane test cell that will give meaningful permeability measurements of diverse membranes.

Major Findings:

The contractors have developed a new membrane test cell to be used primarily as a screening device in membrane development programs. In this device, a single sheet of the test membrane is placed between identical blood and dialysate sides. On each side, fluid flows into an inlet port, through a channel containing polyprophylene mesh and then into an outlet port. The polypropylene mesh in each channel provides membrane support and promotes fluid turbulence. The present design features simplicity of fabrication, ease of assembly and use, and requires a minimum amount of test membrane. Meaningful comparisons of the permeabilities of widely diverse membranes can be obtained with this cell.

Proposed Course:

Work is continuing on developing a process of casting the cells out of epoxy in an effort to reduce the final cost of the device. Test cells should be available in the near future to investigators developing new membranes.

Contractor: New York Medical College

Amount: $119,473

Title: The use of a Special Protein Restricted Diet in Uremia and the Mechanism of its Effectiveness

Objectives:

To evaluate the clinical effectiveness of a modified Giordano-Giovannetti diet in a uremic population and to initiate studies to examine the mechanism of its efficacy.

Major Findings:

Thus far, 33 patients have been studied in comparison of the modified Giordano-Giovannetti with a control diet. Generally, patients with creatinine clearances above 4cc/min responded with lessening of gastrointestinal symptoms, a reduction in blood urea nitrogen and a decrease in transfusion requirement/or an increase in hematocrit. A combination of diet and infrequent dialysis has been used in five patients. It has been possible to maintain these particular patients on hemodialysis every 2-3 weeks.

Proposed course:

An extension of the accummulated experience is planned with a widening of the dialysis-diet group. Nitrogen balance studies will be instituted in selected patients with comprehensive amino acid determinations.

Contractor: North Star Research and Development Institute

Amount: $56,300

Title: Toxic Components in Uremia

Objectives:

To develop a scoring system of reproducible characteristics that occur in uremic animals. Urine will be fractionated by methods that make no presumption of the ultimate identity of the toxin and then infused into anephric animals in an attempt to exacerbate the characteristic patterns. Fractions will then be subfractioniated until the different factors are elucidated.

Major Findings:

The rat was chosen as the experimental animal and a procedure was developed to make the animal anepric while limiting the anesthetic effect. Nine characteristic changes have been reproducibly noted in almost all animals in a pattern which is time related. By utilization of a low protein diet the plateau period is prolonged so that exacerbation after infusion of fractions will be measurable in a simplified manner.

Proposed Course:

Fractionation of urine will begin and infusion of known substances (Urea, uric acid, etc.) as well as other fractions will be evaluated.

Contractor: North Star Research and Development Institute

Amount: $95,400

Title: Development of a New Concept in Membrane Structure for Use in Hemodialysis

Objectives:
Preparation of utra-thin membranes with flow paths tailored to the size of the molecules to be separated. These are membranes composed of compatible copolymers one of which will be leached out producing uniform pores.

Major Findings:
Twelve compatible copolymer mixtures have been developed. Removal of the water soluble component was more difficult than had been anticipated. The membranes thus far developed appear to be less permeable but more selective than cellophane.

Proposed Course:
Variable methods of inducing permeability will be evaluated and the dialysance of these membranes will be evaluated by the differential diffusivity of substances of molecular weights ranging from 100 - 50,000. Special porous film supports will be developed.

Contractor: RAI Research Corporation

Amount: $40,510

Title: A Study of the Synthesis of Hemodialysis Membranes

Objectives:

To apply polymer theory and techniques to the synthesis of superior membranes for hemodialysis purposes.

Major Findings:

The graft polymerization approach had been studied most extensively. Polyvinyl alcohol - polyethylene (0.5 mils) (PVA-PE) grafted membranes were found to have good permeability properties. A preliminary examination also indicated that the grafted membrane had good mechanical properties.

The permeability of only the PVA-PE membrane was evaluated. Dialysis was carried out at 37.5°C using a 0.6% glucose solution. The results were compared to Travenol cellophane. The permeability of the PVA-PE membrane (2.5 mils wet) was somewhat better than cellophane which had a wet thickness of 2 mils.

Contractor: Research Triangle Institute

Amount: $333,234

Title: Medical Registry and Systems Analysis for Chronic Intermittent Dialysis

Objectives:

A patient registry coupled with a medical, engineering and economic analysis of all patients in chronic dialysis has been instituted. The contract provided for a registry of all patients presently maintained with the aid of artificial kidneys or peritoneal dialysis, and will permit a truly comparative evaluation of the effectiveness of the equipment used and of the various methodologies of treatment. The patient registry project aims at a meaningful comparative evaluation of the various facets, steps and methodologies involved in chronic dialysis, from selection of the patient through his medical evaluation once he is being maintained with chronic dialysis. This over-all systems analysis will be undertaken as a cooperative effort with the community service programs in dialysis of the Kidney Disease Control Program of the PHS; it will cover the various dialysis centers of the Veterans Administration Hospitals and all other centers or hospitals engaged in chronic dialysis and willing to cooperate, as well as patients on home dialysis.

Contractor: Research Triangle Institute

mount: $91,122

le: Effect of Uremic and Non-uremic Blood, Urine, and Dialysate Samples and their Fractions on Selected Isolated Enzyme and Biological Systems

Objectives:

This is a new program to determine whether (a) selected pure compounds previously reported to occur in blood, urine or dialysate fluid of uremic patients or fractions thereof can significantly affect appropriately chosen isolated enzyme systems. The ultimate objective is to develop a biological assay for the "uremic toxins" with eventual isolation and identification of these compounds.

Contractor: Research Triangle Institute

Amount: $69,640

Title: Improved Membranes for Hemodialysis

Objectives:

1. Prepare a group of new membranes with high wet strength and superior permeability features. Included are hydroxyethyl starch, polypyrrolidone and diethylene glycol methacrylate membranes.
2. Examine certain graft polymers having component polymers of different polarity.
3. Examine the morphology and characteristics of the above polymers.
4. Examine the permeability properties of the membranes synthesized with emphasis on water content as a screening method for permeability.

Major Findings:

Polypyrrolidinone and 2-hydroxyethyl methacrylate polymers have been successfully formed into membranes of near-unique permeability features. In addition, a method for correlating membrane water content and permeability has been worked out.

Proposed Course:

Continuation of present effort in the membrane area with an additional evaluation cellulose membranes.

Contractor: Stanford Research Institute

Amount: $104,775

Title: Amino Acids, Peptides and Proteoses in Uremia in Man

Objectives:

To investigate the amino acid, peptide and proteose composition of uremic plasma, urine, and dialysate and relate it to that found in normal individuals.

Major Findings:

Significant elevations of 1 and 3 methyl histidine and aspartic acid are found in the plasma of almost all uremic patients (there are other changes found which are not as widespread). Tryptophan and tyrosine are most frequently depressed. Dialysis corrected many of these aberrations although the increased aspartic acid and phenylalanine and decreased taurine were not affected by dialytic therapy. The comparative efficacy of dialysis using Kiil, and Kolff dialysis and of peritoneal dialysis was evaluated and found to be essentially equal with respect to normalization of aberrant amino acid levels. Further, it was found that the amino acids that were extracted into the dialysate had been mobilized from intracellular stores.

Proposed Course:

Study the effect of the Giovanetti-Giordano diet on plasma amino acid levels and attempt to correlate the changes with the clinical status of the patient.

Contractor: Stanford Research Institute

Amount: $117,326

Title: Behavioral and Physiological Correlates of Experimentally Produced Uremic States in Primates

Objectives:

Develop a group of renoprival monkeys upon whom a series of physiological and behavioral tests will be evaluated for their efficacy as indicators of the degree of renal failure.

Major findings:

A colony of squirrel monkeys are azotemic after being subjected to a bilateral 5/6 nephrectomy. The initial post operative period was characterized by a BUN of approximately 80 - 100 mg %. However, after 1 - 2 months it stabilized in the range of 50 - 70 mg %. A series of behaviorial tests have been evaluated only one of which appears to be of value at this time. This test, which utilizes the Wisconsin General Test Apparatus is capable of distinguishing the immediate post operative period from the stable one. The EEG correlated well with the degree of uremia.

Proposed Course:

The study of potentially useful behaviorial and physiologic tests (eg. EEG) in the uremic monkey will be continued. An attempt to correlate changes in the brain enzymes with the degree of uremia will be made.

Contractor: Stanford Research Institute

Amount: $110,450

Title: New Synthetic Membranes for the Dialysis of Blood

Objectives:

Study the partition membrane (as opposed to the diffusing membrane) in so far as molecular structure relates to the solute transport and to investigate the relationship of the polymer surface to thrombogenisis.

Major Findings:

A number of membrane systems, consisting of hydrophilic (utilized primarily for transport properties) and hydropholic (utilized primarily for strength) segments, were synthesized which indicate that solute transport can be achieved with transport rates based on properties other than molecular weight. The hydrophilic segment contributes to both the strength and the separative qualities of the membrane. In vitro coagulation is felt to be unrelated to the _in vivo_ situation and is due to activation of the extrinsic rather than intrinsic coagulation system.

Proposed Course:

An in depth study of the physico-chemical properties of the partition membrane system will be undertaken in an attempt to prove the basic hypothesis upon which the work is based (i.e., the differential solubility of solute in the membrane). The factors involved in surface induced coagulation will be further amplified.

Contractor: The Research Foundation of State University of New York

Amount: $50,737

Title: Developmental Work on Cannulae for Application to Chronic Hemodialysis

Objectives:
To develop basic facts in regard to the plastic shunts, to evaluate new ideas in the laboratory, and finally to institute a clinical evaluation in acute and chronic renal failure patients of promising new features.

Major Findings:
During the two years of research on this contract methods of obtaining fixation of the chronic dialysis cannula as it exits through the skin, and of providing barriers to infection in this area have been explored as well as techniques to accelerate surgical wound healing. A Dacron knitted cloth placed subcutaneously in rabbits as a collar around a prosthesis exiting through the skin was shown to provide firm tissue fixation of the prosthesis and resistance to infection not evident in controls without the subcutaneous collar. Evaluation of this technique for cannulae in chronic dialysis patients is currently being carried out.

Aluminum wire suture which has been reported by some investigators to accelerate wound healing was evaluated in rabbits and dogs and compared to routinely used cat gut suture. No statistical difference was found.

Contractor: Tyco Laboratories, Inc.

Amount: $69,364

Title: Research for Reduced Dialysate Volume in the Artificial Kidney by Development of Specific Lewis Acid Urea Adsorbents

Objectives:

This is a new program designed to:

a) synthesize and evaluate adsorbents or resins containing acidic groups, particularly (OH) with urea bonding properties, for the purpose of selectively removing urea from dialysate solution.

b) determine whether the adsorbent changes the dialysate pH or adds toxic components which may eventually find their way to the patient's blood.

It is proposed to utilize the results of this development to make a preliminary evaluation of the design of an improved low-flow miniaturized dialysis unit.

Contractor: University of California School of Medicine - Wadsworth VA Hospital

Amount: $48,458

Title: Dialysis Instrumentation Research

Objectives:

To devise and/or improve instrumentation for the monitoring of ultrafiltration, blood flow and pressure so as to improve the facility and efficiency of dialysis.

Major Findings:

1. Ultrafiltration Monitoring: This problem has been approached by (a) recording weight changes in the patient and (b) measurement and control of dialysate flow.

Three different bed scales including the Potter, Acme and Brookline bed scales have been evaluated. These scales have been combined with various recording devices for permanent data and rapid derivations of patient weight changes. Data derived in this manner are being correlated with measurement of the transmembrane pressure differential. A negative pressure booster was added to the dialysate compartment. Approximately 600 data pairs have been accumulated on 20 different patients relating weight changes to transmembrane pressures. These data are currently undergoing extensive analysis.

A Starling pneumatic pump has been modified in an effort to control dialysate flow. Gravimetric techniques are also being investigated in attempts to control this parameter.

2. Hemodynamic Control: Piezo-electric and photoelectric transducers have been evaluated with respect to recording pulse contours. Continuous modification is necessary before a satifactory sensing and recording device will be realized. A central system is being completed in the dialysis unit that will allow monitoring of cardiac rates of patients on dialysis. Several heart rates can be recorded simultaneously on one chart.

Venous and arterial line pressure monitoring have been investigated by various techniques with alarm devices introduced into the system.

Blood flow measurements have been limited to evaluation of an electromagnetic flowmeter; but, an ultrasonic flowmeter, operating on the Doppler principal, will be evaluated.

Contractor: University of California, Los Angeles

Amount: $38,028

Title: Effect of low protein Diet on Dialysis Requirement

Objectives:

To test the premise that a low protein diet of high biological quality can decrease the dialysis requirement of chronic uremic patients.

Contractor: University of Minnesota (Contract work performed at Cornell University Medical Center because of change of location of principal investigator)

Amount: $81,800

Title: A New Disposable Artificial Kidney

Objectives:

To use economical mass-production methods of plastic manufacture to produce virtually identical rigid membrane supports which will equally distribute blood flow through modular short parallel flow path dialyzers in order to develop an efficient disposable artificial kidney.

Major Findings:

Various methods of assembly have been investigated and it has been found that the support plates may be held together satisfactorily with metal bands. Flow and dialysis characteristics of the unit are being determined and modifications are being made to optimize these parameters.

Proposed Course:

Means for developing large numbers of these dialyzes at high quality and low cost will be explored while studies are underway to further characterize and improve the performance of the dialyzer.

Contractor: University of North Carolina

Amount: $34,650

Title: Antithrombogenic Surfaces: platelet-Interface Reaction

Objectives:

This is a new program directed at development of hypothrombogenic surfaces through study of a variety of materials and their ability to attract platelets and initiate clotting. Those factors in blood platelets, plasma, erythrocytes or leukocytes responsible for formation of white thrombi will be identified.

Contractor: University of Pennsylvania

Amount: $29,304

Title: Development of Testing of a Disc Cone Dialyzer

Objectives:

Develop a clinical model of a radial flow dialyzer utilizing the cone membrane support in order to provide efficient membrane scrubbing, uniform distribution of blood and dialysate, optimal combination of blood flow, blood volume, blood pressure drop and blood path thickness as well as minimizing the change of membrane rupture.

Contractor: University of Pennsylvania

Amount: $17,878

Title: Batch Dialyses Testing of Ioplex Membranes

Objectives:

A study of the transport capabilities of polyelectrolyte membranes in a batch diffusion test cell.

Major Findings:

The transport characteristics of these membranes were equal to, or less than, the standard cuprophane membrane. However, under the condition of ultrafiltration, the membranes were superior to cuprophane in that water flux was increased by a factor of 12.

Proposed Course:

These data are being utilized in an investigation of hemodiafiltration.

Contractor: University of Pennsylvania

Amount: $87,000

Title: Blood Purification by Ultrafiltration and Reconstitution

Objectives:

Evaluation of the permeability of the ioplex membranes to solutes ranging from 500 - 10,000 in molecular weight is being carried out. A prototype dialyzer utilizing ultrafiltration and reconstitution procedures is being developed.

Major Findings:

During ultrafiltration of protenaceous fluids, a cake of protein built up on the membrane-blood interface which impeded effective transport. Analysis of the etiology of this phenomenon indicates that the size of the channels in which the blood flows is a critical factor. In light of this finding, the prototype dialyzer is envisioned as having flat plate supports with micro-grooves in order to decrease the layering of protein. The first model has been developed and the protein layering has been reduced to a great extent.

Proposed Course:

Effort will continue in the direction of the development of a grooved flat plate dialyzer. In vivo evaluation of the prototype, on dogs, is anticipated.

Contractor: University of Pisa

Amount: $22,242

Title: Study on the Role of Guanidines and Related Compounds in Producing Uremic Symptoms

Objectives:

The role of guanidines and related compounds in producing the uremic manifestations of peripheral neuropathy, glucose intolerance, and hemolytic-bleeding tendencies will be determined. These compounds will be measured in uremic humans. Subsequently, they will be given parenterally to mononephrectomized dogs and the effect on nerve conduction times, glucose tolerance tests, and a variety of hematologic parameters determined. Bone marrow tissue cultures to which guanidines are added will be observed for their rates of metabolic activity.

Contractor: University of Pisa

Amount: $26,985

Title: Diet in Chronic Uremia

Objectives:

To study the effect of the amount and quality of dietary proteins on uremic symptomatology and the long term effects of a high colorie, low protein diet on protein metabolism. This is a new program.

Contractor: University of Tennessee

Amount: $49,700

Title: Mass Transfer During Dialysis

Objectives:

The objective is to develop new mathematical models and to utilize existing models to simulate and describe the kinetic aspects of mass transfer during dialysis. *In vitro* tests of the models will include studies with various dialyzers and membranes. *In vivo* testing using dogs and humans will be included subsequently.

Contractor: University of Utah

Amount: $213,693

Title: Development and Testing of Efficient but Less Costly Artificial Kidneys for Hospital and Home Use

Objectives:

The objective is to develop a new artificial kidney designed to be highly efficient and low in cost. A wide coil using cellophane tubing will be investigated for ease of manufacture, ease of sterilization, and high permeability. In addition, improved safety devices and physiologic monitors which include a blood leak detector, conductivity meters, blood pumps, automated proportioning pumps to mix electrolyte-water solutions, and blood pressure recorders will be developed. A hypobaric, recirculating dialysate kidney module will be built and its dialysis and ultrafiltration potential characterized. Finally, an integrated dialyzing unit will be assembled and will undergo clinical testing.

Contractor: University of Washington

Amount: $13,332

Title: Development of a Bacteriologically Safe Peritoneal Access Device

Objectives:

Development of a safe and reliable peritoneal access device.

Major Findings:

The investigation has centered around the development of a peritoneal access device which would establish easy access to the peritoneal cavity for the purpose of intermittent peritoneal lavage. Using dogs as the experimental animal, the investigators have developed a silastic catheter surrounded by a dacron cuff at the skin exit site. The cuff allows connective tissue ingrowth and acts as a bacterial seal. A subcutaneous tunnel, anatomically separating the skin exit site from the entrance into the peritoneal cavity, further minimizes the chances of infection. The results obtained to date with animals have led to its evaluation in patients. Clinical evaluation has not been completed.

Contractor: University of Washington

Amount: $64,068

Title: Development of a Low-Cost Home Peritoneal Dialysis System

Objectives:

The investigators are seeking to develop an inexpensive, simple, bacteriologically safe peritoneal dialysis fluid supply system for home use. Commercially available depth filters will be evaluated for their capacity to remove particulate matter, pyrogens, and other noxious substances from tap water. Use of heat will be investigated for the sterilization of filtered water. Success of these efforts and the development of a proportioning device to mix electrolyte and sterile water, should allow the implementation of an automated dialysate production process in the home.

Contractor: University of Washington

Amount: $75,150

Title: Fluid Mechanics and Mass Transfer Studies, Hemodialyzers and Hemodialyzer Optimization Studies

Objectives:

The objectives are to investigate fluid dynamics and mass transfer studies necessary to design and fabricate an optimized hemodialyzer.

Major Findings:

In vivo tests of the Babb-Grimsrud nickel foam dialyzer have confirmed earlier data which indicated that its dialyzing performance is superior to the 1m^2 Kiil Dialyzer. As a result of testing, several modifications have been made and are being made with the goal of providing a dialyzer which can be produced in quantity and which will retain its mechanical and structural integrity under clinical conditions.

Equations and graphs have been developed to describe the operating characteristics of a hemodialyzer. These results can be used to aid in the design of dialyzers and in determining proper dialyzer operating conditions. New testing procedures have been formulated and data obtained for the determination of solute diffusivities and membrane permeabilities. A method for obtaining mass-transfer data from *in vivo* experiments with uremic patients is now being implemented.

Proposed Course:

Clinical testing will continue. The Babb-Grimsrud dialyzer should be produced in quantities sufficient for testing in at least a limited number of clinical centers. Optimization of the dialyzer will continue. Studies on fluid mechanics and mass transfer will continue.

Contractor: University of Washington School of Medicine

Amount: $108,943

Title: Cannula Research

Objectives:
To evaluate cannula systems currently being used in chronic hemodialysis and to develop systems of improved design.

Major Findings:
Teflon and silastic cannula designs which are modifications of the original Quinton-Scribner arteriovenous cannula have been fabricated and tested in animals and in acute human trials. New techniques of attaching cannulae to animal vessels have been experimented with using a Dacron knitted cloth as a junction material. A new design coupler for easier connection of the patient to the artificial kidney has been developed in conjunction with the Becton, Dickinson and Company, and is undergoing clinical testing. Different lengths and diameters of needles are being experimented with in patients with arteriovenous fistulae to minimize the difficulty and complication encountered in the repeated intravenous needle placement in these patients.

Proposed Course:
Further animal and human trials of new cannula designs are proposed. Development of techniques for patients with arteriovenous fistulae to allow this technique to be used by the home dialysis patients will be continued.

Contractor: Western Gear Corporation

Amount: $195,280

Title: Artificial Kidney Development Program

Objectives:

The overall objectives are two-fold. The first is to modify the Kiil Dialyzer in order to improve efficiency, reduce assembly time, improve sealing, and reduce size and weight. The second is to develop new dialyzer designs.

Major Findings:

Emphasis has been placed on the first objective with the following results. A lighter weight Kiil board has been produced which is about 40% lighter than existing polypropylene boards and a clamping system has been designed which is about 25% lighter than the system currently being used. Construction of the membrane support boards is a composite of a polyester resin envelope around a polyurethane foam center.

A membrane support has been fabricated using a multiple point support configuration resulting from cutting a second set of grooves perpendicular to those in the existing Kiil design. This design is undergoing clinical trials and appears to be as efficient in a one-layer configuration as the standard Kiil is in a two-layer configuration.

Investigation of a multiple layer unit which is smaller and lighter than existing units is underway.

Presterilized membranes assembled and ready for use have been developed and are being tested clinically. Results suggest that they will provide convenience especially in the home dialysis situation.

Proposed Course:

It is proposed to complete clinical trials of the lighter Kiils, of the single layer multiple support Kiil and of the presterilized membrane. Modifications, if needed, will be made and units will be produced in quantities sufficient for distribution for additional testing and application.

THE CLINICAL FIELD STUDIES UNIT
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

SUMMARY

The Clinical Field Studies Unit of the National Institute of Arthritis and Metabolic Diseases located in Phoenix, Arizona, initiated a long-term prospective program in which the development and progression of the various manifestations and complications of diabetes, arthritis, and gallbladder disease will be studied and evaluated on the Gila River Indian Reservation for ten to fifteen years. Various conditions and factors, including hereditary, environmental, dietary, and anthropological aspects, that may have a bearing on the frequency and severity of these diseases and/or their complications, are being investigated and selected findings contrasted with similar studies performed on other populations with different characteristics or environment.

An attempt is being made to examine approximately 3500 Indians living on the Sacaton Service Unit area of the Gila River Indian Reservation every two years for a minimum of ten years. The basic biennial examinations performed on all respondents consist of medical history, physical examination, biochemical tests, electrocardiograms, soft-tissue radiographs, joint radiographs and fundus photographs. In addition, various special tests, examinations and interviews are performed on selected subsamples of the population to obtain additional data and information which will be correlated and analyzed in conjunction with the basic data to give a more complete picture or to test various hypotheses.

The basic examinations are carried out in a portable clinic building adjacent to the outpatient wing of the PHS Indian Hospital at Sacaton, Arizona. Some examinations and interviews, however, are performed in a mobile clinic van or at the respondent's home, depending upon the individual study and circumstance.

Storage and retrieval of accumulating data has been handicapped by the unavailability of adequate automatic data processing facilities. Arrangements are presently being negotiated for computer support and when available the evaluation and analysis of the data should be expedited.

Although the overall program of the Unit has been divided into various projects depending upon the major disease or type of study, the projects are integrated and carried out simultaneously.

Demographic and Genetic Variation

Probably the most fundamental facet of the entire spectrum of studies being undertaken on the Gila River Reservation is the prosaic one of continuously updating and keeping current the demographic and geneological data. This is essential, since without a knowledge of the basic population with which we are working, it would be impossible to determine what proportion of the community we had examined or to calculate meaningful ommunity rates.

pled with this phase of the overall investigative program, historical and genetic data are collected which will be used in computer programs presently being written to assess consanguinity, mating patterns, genetic drift, etc., in the various communities on the reservation.

Arthritis and Rheumatism

While the basic data has been collected concerning arthritis and rheumatism on all Indian respondents during this past year, the main analysis was based on radiographs and data from the 1963 and 1965 surveys. This showed that, among individuals with no clinical or radiological evidence of rheumatoid arthritis, a higher proportion of those with a positive test for rheumatoid factor developed changes associated with rheumatoid arthritis than those with a negative test. The difference, however, was not significant in the two-year period. Similar data from the 1967 examinations on the same individuals is now being compiled to assess the four-year prognostic value of rheumatoid factor tests.

Review of radiographs of the spine indicated that hyperostotic spondylosis is very common in the present day Pima population. This condition has been prevalent in the southwestern Indians for many centuries since radiographs of prehistoric Indian remains, taken at our suggestion by the University of Arizona, Department of Anthropology, have shown the condition very clearly.

Gallbladder Disease

Cholelithiasis is extremely common in the Pima Indians. Review of the Sacaton Hospital records revealed that six percent of the males and 31 percent of the females between the ages of 15 and 74 had definite evidence of gallbladder disease as shown by a history of cholecystectomy or gallstones on x-ray examination. Silent gallstones are extremely common since when cholecystograms were done on those without a positive history of gallbladder disease, a total of 38 percent of the males and 68 percent of the females were shown to be affected. When the present prevalence studies are completed, additional studies will be initiated to assess the importance of various factors shown to be associated with those having the disease.

Diabetes Mellitus

This is probably the most important disease affecting the Indians of the Gila River Reservation. The present studies have been primarily directed to ascertaining the frequency and importance of the carbohydrate abnormality and the various chronic complications and conditions associated with them. These studies have demonstrated that the disease and its complications are extremely prevalent in the population but analysis of association of diabetes with various demographic, genetic and environmental parameters will have to wait until the computer masterfile and retrieval system is operational since the mass of data is already too voluminous for hand tabulation and calculations.

Diet and Nutrition

A prospective study on women aged 25-44 which is the age and sex group with the highest incidence of diabetes has been initiated to evaluate possible influence of diet on subsequent development of diabetes and cholelithiasis.

Geographic and Racial Variation

In addition to the studies on the Gila River Reservation, the Unit has also participated in surveys sponsored by the Division of Indian Health and also by the National Institute for Dental Research to obtain information on other tribes of Indians with which to compare data from the Gila River Reservation.

Probably one of the most significant studies was done at the Sherman Institute, a Bureau of Indian Affairs Boarding School, Riverside, California. Glucose tolerance tests were conducted on students before and after the summer vacation. Pima children who lived on the reservation during the summer vacation gained more weight than the Pima children who lived off the reservation and also had a significant increase in the mean plasma glucose value. A similar, but less marked change, was also noted in the Papago Indians.

Serial No. NIAMD-EFS-1(c)
1. Epidemiology & Field Studies Br.
2. Clinical Field Studies Unit
3. Phoenix, Arizona

PHS-NIH
INDIVIDUAL PROJECT REPORT
July 1, 1967 through June 30, 1968

PROJECT TITLE: GEOGRAPHIC AND RACIAL VARIATION IN HEALTH AND DISEASE

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Dr. Thomas A. Burch

OTHER INVESTIGATORS:	Dr. Peter H. Bennett Dr. Robert E. Henry Dr. R. Gordon Senter Dr. Baruch S. Blumberg Dr. Donald Richey (DIH) Dr. Jerry D. Niswander (NIDR)
COOPERATING UNITS:	Division of Indian Health (PHS) Bureau of Indian Affairs National Institute of Dental Research Institute for Cancer Research, Philadelphia

MAN YEARS:

Total:	1/4
Professional:	1/8
Other:	1/8

PROJECT DESCRIPTION:

Objectives:

To determine the frequency of diabetes, arthritis, rheumatoid factor and other conditions including various genetic parameters in various populations and to compare such findings with similar observations on the Gila River Indian Community.

Methods:

Cross sectional surveys on the above conditions and factors will usually be performed in collaboration with surveys conducted by other organizational units and will be based, insofar as practical, on the procedures and techniques used in the more detailed studies on the Gila River Indian Community

Major Findings:

1. Changes in plasma glucose values at Sherman Institute. Modified glucose tolerance tests were performed on 84 Pima and 88 Papago students before

and after the 1967 summer vacation at the Bureau of Indian Affairs Boarding School at Riverside, California. The mean plasma glucose of the Pima students increased from 83.3 mg% to 93.3 mg% ($p<0.01$) while that of the Papago students only increased from 86.4 mg% to 90.6%--a difference that was not statistically significant.

The increase was most marked on the Indian children who resided on their respective reservations during the summer and was associated with an increase in percentage of desirable weight.

TWO HOUR PLASMA GLUCOSE VALUES OF INDIAN STUDENTS AT SHERMAN INSTITUTE

CATEGORY	NUMBER	AVERAGE MARCH 2 HR. GLUCOSE	AMERAGE SEPTEMBER 2 HR. GLUCOSE	P. VALUE
On Reservation Pima	48	82.3	95.0	<0.01
Off Reservation Pima	35	85.0	91.3	0.20
On Reservation Papago	56	81.6	89.4	<0.01
Off Reservation Papago	27	96.3	93.0	0.60

PERCENTAGE OF DESIRABLE WEIGHT OF INDIAN STUDENTS AT SHERMAN INSTITUTE

CATEGORY	NUMBER	AVERAGE MARCH % D. WT.	AVERAGE SEPTEMBER % D. WT.	P. VALUE
On Reservation Pima	47	107.7	116.4	0.001
Off Reservation Pima	34	115.2	112.0	0.10
On Reservation Papago	56	105.4	108.4	0.05
Off Reservation Papago	27	114.0	115.2	0.70

2. Prevalence of diabetes on the Papago Indian Reservation. A total of 644 Papago Indians have now had modified glucose tolerance tests in conjunction with the National Institute of Dental Research survey on the Papago Reservation. The frequency of diabetes is lower in all sex and age groups in the Papago than in the Pima except males age 15-24 and this difference is not significant.

		PAPAGO			PIMA		
SEX	AGE GROUP	EXAMINED	DIABETIC		EXAMINED	DIABETIC	
Male	05-14	130	1	1%	248	12	5%
	15-24	29	4	14%	161	14	9%
	25-34	36	3	8%	85	20	24%
	35-44	36	8	22%	91	33	36%
	45-54	15	1	7%	76	36	47%
	55-64	13	4	31%	80	40	50%
	65+	17	2	12%	110	47	43%
	TOTAL	276	23	8%	851	202	24%

		PAPAGO			PIMA		
SEX	AGE GROUP	EXAMINED	DIABETIC		EXAMINED	DIABETIC	
Female	05-14	170	0	0%	278	21	8%
	15-24	46	0	0%	218	41	19%
	25-34	68	5	7%	164	57	35%
	35-44	41	4	10%	152	75	49%
	45-54	20	2	10%	106	59	56%
	55-64	14	7	50%	97	69	71%
	65+	9	4	44%	86	49	57%
	TOTAL	368	22	6%	1101	371	34%
TOTAL		644	45	7%	1952	573	29%

3. Serum Albumin Polymorphism. Determination of albumin variants has been completed on the plasma from the first Sherman Institute survey by Dr. Baruch S. Blumberg of the Institute for Cancer Research, Philadelphia. The approximate frequency of three of the variants in the five major tribes attending the Sherman Institute were as follows:

TRIBE	A/AUSTRALIA	A/NASKAPI	A/MEXICO
Navajo	33%	6%	1%
Apache	36%	3%	7%
Papago	30%	0%	3%
Pima	35%	0%	7%
Hopi	23%	1%	0%

Significance of Research:

These studies will enable comparisons to be made between various populations with different characteristics or environment and permit testing hypothesis developed in studies on the Gila River Reservation.

Proposed Course:

Short term surveys will be conducted on other population groups whenever possible

HONORS AND AWARDS: None

PUBLICATIONS: None

Serial No. NIAMD-EFS-2(c)
1. Epidemiology & Field Studies Br.
2. Clinical Field Studies Unit
3. Phoenix, Arizona

PHS-NIH
INDIVIDUAL PROJECT REPORT
July 1, 1967 through June 30, 1968

PROJECT TITLE: DEMOGRAPHIC AND GENETIC VARIATION IN THE GILA RIVER INDIAN COMMUNITY

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Dr. Thomas A. Burch

OTHER INVESTIGATORS:
Dr. Peter H. Bennett
Dr. Robert E. Henry
Dr. Leonard J. Comess
Dr. R. Gordon Senter
Dr. G. Albin Matson
Dr. Herbert F. Polesky
Mr. Dwight A. Rokala
Dr. Arthur G. Steinberg

COOPERATING UNITS:
Division of Indian Health (PHS)
Minneapolis War Memorial Blood Bank
Case Western Reserve University
University of Utah

MAN YEARS:
Total: 1 1/2
Professional: 1/2
Other: 1

PROJECT DESCRIPTION:

Objectives:

To assess the demographic-geneological factors affecting the distribution of genetic parameters and the various disease conditions being studied in the Gila River Indian Community.

Methods:

1. Establish and maintain a demographic and geneological census of the Gila River Indian Community in conjunction with the prospective studies on arthritis and diabetes.

2. Arrange for the assessment of as many different genetic markers as possible. Those currently being determined are:

a. Blood groups.
b. Serum albumin variants.
c. Group specific component.
d. Transferrin.
e. Haptoglobin.
f. Gm groups.

3. Evaluate the association of genetic, demographic, and environmental parameters with arthritis, diabetes and associated conditions in the Gila River Indian Community.

Major Findings:

1. The population of the Sacaton Service Unit area of the Gila River Indian Reservation consisted of 3337 Pima-Papago Indians age 5 years and over. The age and sex breakdown is as follows:

AGE GROUP	MALES	FEMALES	TOTAL
05-09	288	303	591
10-14	265	268	533
15-24	365	332	697
25-34	216	207	423
35-44	171	174	345
45-54	131	127	258
55-64	124	114	238
65-74	102	64	166
75+	60	28	88
TOTAL	1722	1617	3339

2. Polesky and Rokala of the Minneapolis War Memorial Blood Bank have done starch gel electrophoresis on 1528 serum specimens from Pima Indians on the Gila River Reservation. In the Pima population an albumin variant, migrating slower than the usual type, was encountered in 107 individuals. This was tentatively designated as albumin Pima although it is probably the same as Albumin Mexico of Blumberg. One Pima was encountered who was heterozygous for the Algonkian variant and the usual type of serum albumin.

ALBUMIN PHENOTYPE	NUMBER	PERCENT
A/A	1421	92.90
A/P	100	6.50
P/P	7	0.50
A/A1	1	0.01
TOTAL	1529	

Significance of Research:

Study of multiple genetic parameters among the Gila River Indians affords unique opportunities in research of human population ecology through the combined application of genetic and epidemiological methodologies.

In addition to genetic characterization of representative samples from each of the several communities on the Reservation, it will be possible to relate the genetic data to the operation of random and nonrandom social processes within and between communities. As such processes are delimited, through application of both demographic and geneological matrices, additional significant means for the subdivision and analysis of the data should become available.

Proposed Course:

The demographic and geneological data will be kept current by interviewing respondents during their clinic visits and by periodic re-surveys. The data from the NIAMD studies will be combined with other available data and analyzed by means of computer programs.

HONORS AND AWARDS: None

PUBLICATIONS:

Matson, G. A., Burch, T. A., Polesky, H. F., Sutton, H. E., and Robinson, A.: Distribution of hereditary factors in the blood of Indians of the Gila River, Arizona. Amer. J. Phys. Anthrop., (In press).

Serial No. NIAMD-EFS-3(c)
1. Epidemiology & Field Studies Br.
2. Clinical Field Studies Unit
3. Phoenix, Arizona

PHS-NIH
INDIVIDUAL PROJECT REPORT
July 1, 1967 through June 30, 1968

PROJECT TITLE: PROSPECTIVE STUDY OF THE NATURAL HISTORY OF ARTHRITIS AND RHEUMATISM ON THE GILA RIVER INDIAN COMMUNITY

PREVIOUS SERIAL NUMBER: Same

PRINCIPAL INVESTIGATOR: Dr. Peter H. Bennett

OTHER INVESTIGATORS:
Dr. Thomas A. Burch
Dr. Leonard J. Comess
Dr. Robert E. Henry
Dr. R. Gordon Senter
Dr. John L. Decker (AR)

COOPERATING UNITS:
Arthritis & Rheumatism Branch, NIAMD
Division of Indian Health, PHS

MAN YEARS:

Total	2
Professional	1/2
Other	1 1/2

PROJECT DESCRIPTION

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthrosis, ankylosing spondylitis and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predicative and prognostic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods:

1. The following tests and examinations will be repeated on all Indians 5 years of age or more living on the Sacaton Service Unit portion of

the Gila River Reservation.

- a. Clinical interview.
- b. Physical examination.
- c. Radiographs of hands, feet, and cervical spine. Males over age 14 and females over age 44 will have a pelvis film taken.
- d. Laboratory determinations: rheumatoid factor, uric acid, cholesterol, creatinine and alkaline phosphatase.

Major Findings:

1. Prognostic Significance of Positive Test for Rheumatoid Factor. In order to determine the significance of a positive test for rheumatoid factor in the absence of rheumatoid arthritis a group of 55 subjects with a positive test for RF and an age-sex matched group of controls with a negative test for RF in 1963 were re-examined in the period between March 1, 1965 and 1967 for characteristics associated with rheumatoid arthritis.

CRITERION	SEROPOSITIVE SUBJECTS (55)			CONTROL SUBJECTS (54)		
	Same or Improved Number	Deteriorated		Same or Improved Number	Deteriorated	
		No.	%		No.	%
Clinical RA:						
Grade 1+	38	17	30.9	41	13	24.1
Grade 2+	49	6	10.9	49	5	9.3
Morning Stiffness	42	13	23.6	45	9	16.7
Pain on Motion	49	6	10.9	48	6	11.1
Swelling	50	5	9.1	51	3	5.5
Subcutaneous Nodules	55	0	0.0	54	0	0.0
Radiological Erosive Changes in Hands & Feet	43	10	18.9	43	6	12.2

In general the status of the seropositive subjects deteriorated to a greater extent than that of the control subjects. In no instance, however, was the difference statistically significant. Both groups will be examined at two-year intervals to determine if significant differences emerge.

2. Hyperostotic spondylosis. This is a disease of the spine characterized radiologically by osteophyte formation, bridging of intervertebral spaces, ossicle formation and new bone formation. Radiographs of the spines of 214 males and 256 females over the age of 15 were reviewed for evidence of hyperostotic spondylosis. Eighty-eight or 41% of the males and 56 or 22% of the females had definite disease. All cases were at least 35 years of age.

Oral glucose tolerance tests were done on 394 of the subjects. Sixty-six percent of the diabetic males had hyperostotic spondylosis compared to 48% of the nondiabetic males. The corresponding values for females were 33% of diabetics and 26% of nondiabetics.

MALES

AGE	DIABETICS	NUMBER WITH HS	PERCENT	NONDIABETICS	NUMBER WITH HS	PERCENT
35-44	11	3	27	16	2	13
45-54	20	14	70	11	4	36
55-64	14	8	57	17	15	88
65-74	23	20	87	16	8	50
TOTAL	68	45	66	60	29	48
			FEMALES			
35-44	22	2	9	21	1	5
45-54	29	10	34	15	3	20
55-64	31	11	35	12	5	42
65-74	25	12	48	18	8	45
TOTAL	107	35	33	66	17	26
GRAND TOTAL	165	80	48	126	46	36

3. Metacarpophalangeal Cysts. The hand x-rays of the Pima Indians examined in 1963 were re-evaluated for the presence of cytic changes in the MCP joints.

A group of 47 males who had the most numerous and severe cysts were compared to an age matched group of males without MCP cysts. There was a significant increase in morning stiffness among the group with cysts ($p<.01$) but this was not true of any of the other A.R.A. criteria examined, either singly or in combination and we conclude that the majority of the cysts seen are not associated with RA. The group with cysts had higher frequencies of Heberden's nodes, limitation of motion in the peripheral joints, radiological evidence of OA in the hands, or feet. Each of these associations was statistically significant ($p <0.01$). It was concluded that the majority of the cystic lesions seen were associated with OA.

Significance of Research:

This study is designed to identify and describe the arthritides which occur frequently in a well defined community.

Rheumatoid arthritis is responsible for considerable morbidity and identification of factors which are related to the development of the arthritis would make the use of prophylactic measures a realistic goal. The etiology of the majority of the forms of degenerative arthritis remains obscure, but description of their natural history is expected to yeild etiologic clues worthy of further exploration.

Proposed Course:

The collection of data from the Pima Indians at biennial intervals will be continued over a period of years so that the development and rate of progression of the various rheumatic diseases may be followed.

The prevalence of rheumatic diseases in the children and young adults will be determined and associated factors will be sought.

The relationship of positive bentonite and sheep cell agglutination tests to the development of rheumatoid arthritis and other diseases will be investigated and the significance of positive bentonite and sheep cell agglutination alone and in combination will be determined.

Studies of osteoarthrosis will continue in the hope that unknown genetic and environmental factors will be identified. The frequency of ankylosing spondylitis among Blackfeet and Pima Indian females will be determined.

HONORS AND AWARDS: None

PUBLICATIONS:

Bennett, P. H., and Burch, T. A.: New York symposium on population studies in the rheumatic diseases: New diagnostic criteria. Bull. Rheum. Dis. 17: 453-458, Apr. 1967.

O'Brien, W. M., Bennett, P. H., Burch, T. A., and Bunim, J. J. (deceased): A genetic study of rheumatoid arthritis and rheumatoid factor in Blackfeet and Pima Indians. Arthritis Rheum. 10: 163-179, June 1967.

Bennett, P. H., and Burch, T. A.: The genetics of rheumatoid arthritis. In Bennett, Peter H., and Wood, Philip H. N. (Eds.): Population Studies of the Rheumatic Diseases. New York, Excerpta Medica Foundation, 1968, pp. 136-145.

Bennett, P. H., and Burch, T. A.: Osteoarthrosis in the Blackfeet and Pima Indians. In Bennett, Peter H., and Wood, Philip H. N. (Eds.): Population Studies of the Rheumatic Diseases. New York, Excerpta Medica Foundation, 1968, pp. 407-412.

Bennett, P. H., and Burch, T. A.: The epidemiological diagnosis of ankylosing spondylitis. In Bennett, Peter H., and Wood, Philip H. N. (Eds.): Population Studies of the Rheumatic Diseases. New York., Excerpta Medica Foundation, 1968, pp. 305-311.

Bennett, P. H., and Burch, T. A.: Serum uric acid and gout in Blackfeet and Pima Indians. In Bennett, Peter H., and Wood, Philip H. N. (Eds.): Population Studies of the Rheumatic Diseases. New York., Excerpta Medica Foundation, 1968, pp. 358-362.

Bennett, P. H., and Burch, T. A.: Rheumatoid factor in the Blackfeet and Pima Indians. In Bennett, Peter H., and Wood, Philip H. N. (Eds.): Population Studies of the Rheumatic Diseases. New York., Excerpta Medica Foundation, 1968, pp. 192-199.

Burch, T. A.: Mortality from rheumatoid arthritis in the United States, 1959-1961. In Bennett, Peter H., and Wood, Philip H. N. (Eds.): Population Studies of the Rheumatic Diseases. New York, Excerpta Medica Foundation, 1968, pp. 39-42.

Editor: Bennett, Peter H., and Wood, Philip H. N. (Eds.): Population Studies of the Rheumatic Diseases. New York, Excerpta Medica Foundation, 1968, 504 pp.

Bennett, P. H., and Burch, T. A.: The epidemiology of rheumatoid arthritis. In Bland, J. (Ed.): The Medical Clinics of North America. Philadelphia, Pa., W. B. Saunders Co., 1968, pp. 479-491.

Serial No. NIAMD-EFS-4(c)
1. Epidemiology & Field Studies Br.
2. Clinical Field Studies Unit
3. Phoenix, Arizona

PHS-NIH
INDIVIDUAL PROJECT REPORT
July 1, 1967 through June 30, 1968

PROJECT TITLE: GILA RIVER INDIAN COMMUNITY GALLBLADDER STUDY

PREVIOUS SERIAL NUMBER: Same

PRINCIPAL INVESTIGATOR: Dr. Peter H. Bennett

OTHER INVESTIGATORS:
Dr. Thomas A. Burch
Dr. Leonard J. Comess
Dr. John S. Fordtran
Dr. Franz J. Ingelfinger

COOPERATING UNITS:
Division of Indian Health, PHS
Southwestern Medical School
Boston University

MAN YEARS:
Total: 1/2
Professional: 1/4
Other: 1/4

PROJECT DESCRIPTION:

Objectives:

1. To determine the age and sex-specific prevalence of cholelithiasis in a random sample of Pima Indians living in the Sacaton Service Unit area of the Gila River Indian Reservation.

2. To identify personal and environmental factors associated with cholelithiasis.

Methods:

1. Fifty male and fifty female Pima Indians in each of the six decades between 15 and 74 were selected using random numbers from the NIAMD Demographic Survey of the Gila River Indian Reservation.

2. The Sacaton Hospital records of all selected subjects were reviewed for history of previous gallbladder x-rays or surgery.

3. A cholecystogram will be performed on all respondents without specified contraindications.

Major Findings:

1. Since February 1, 1967, a total of 192 Pima Indians have been evaluated for gallbladder disease at the CFSU Clinic at Sacaton. Sixty of these had previously documented gallbladder disease and oral cholecystograms were obtained on 132. Fifty-three or 40 percent of the latter group without known disease either had nonfilling gallbladders or evidence of stones.

2. The following table compares the age sex specific frequencies of definite gallbladder disease as shown by reviewing the Sacaton Hospital records and by specific evaluation for gallbladder disease at the CFSU Clinic.

		HOSPITAL RECORDS			CFSU CLINIC EVALUATION		
			With Definite GB Disease			With Definite GB Disease	
Sex	Age Group	Total Reviewed	No.	%	Total Examined	No.	%
Males:							
	15-24	43	0	0.0	5	0	0.0
	25-34	47	2	4.3	6	0	0.0
	35-44	48	0	0.0	9	1	11.1
	45-54	48	2	4.2	10	4	40.0
	55-64	44	8	18.2	11	6	54.5
	65-74	50	5	10.0	15	10	66.7
	TOTAL	280	17	6.1	56	21	37.5
Females:							
	15-24	48	1	2.1	11	1	9.1
	25-34	45	14	31.1	18	13	72.2
	35-44	52	24	46.2	33	22	66.7
	45-54	46	17	37.0	28	19	67.9
	55-64	48	13	28.1	19	12	63.2
	65-74	55	23	41.8	27	25	92.6
	TOTAL	294	92	31.3	136	92	67.6

3. The data shown above indicate that gallbladder disease in Pima Indians is exceedingly prevalent in both sexes and, although clearly age related in males is less clearly so in females. Thus, although both sexes in the Pima are extremely prone to develop gallbladder disease, some additional factors in the females favor an earlier onset of disease.

4. Further analyses on the above group have indicated that number of pregnancies in Pima females is unassociated with gallbladder disease as is diabetes in both sexes. Among females only, those with gallbladder disease had a significantly higher mean percent desirable weight than those without disease.

5. Review of the hospital records of 205 consecutive admissions of Pima Indians to the Sacaton Hospital indicated a prevalence of clinic gallbladder disease similar to that found by reviewing the hospital records of the random sample. This indicates that the hospital population at Sacaton is more representative of the community population than found in most hospitals.

Significance of Research:

The etiology of gallbladder disease is unclear. Personal and environmental factors such as diet, number of pregnancies, and obesity are said to be of importance in producing gallstones. Genetic influence is suggested by the difference in frequency of the disease at autopsy in Negroes and Caucasians. Further studies in populations with varying frequencies of gallbladder disease are needed so that the relative importance of various associated factors may be assessed. The Pima Indians, with a high prevalence of gallbladder disease, readily accessable medical records, and a stable population, are an ideal group upon which to perform such a study.

Proposed Course:

1. Cholecystograms have been made on approximately 132 members of the above sample, who had not already had gallstones demonstrated by x-ray or surgery. This will be continued to determine the frequency of asymptomatic gallstones.

2. The hospital records of 205 Pima or Papago Indians older than 14 years of age admitted to the Sacaton Hospital are being reviewed for evidence of documented gallbladder disease. This will be continued until a total of 600 records have been reviewed to ascertain if the hospital sample is representative of the population as shown by the random sample.

HONORS AND AWARDS: None

PUBLICATIONS:

Comess, L. J., Bennett, P. H., and Burch, T. A.: Clinical gallbladder disease in Pima Indians: Its high prevalence in contrast to Framingham, Massachusetts. New Eng. J. Med. 277: 894-898, Oct. 1967.

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Serial No. NIAMD-EFS-5(c)
1. Epidemiology & Field Studies Br.
2. Clinical Field Studies Unit
3. Phoenix, Arizona

PHS-NIH
INDIVIDUAL PROJECT REPORT
July 1, 1967 through June 30, 1968

PROJECT TITLE: PROSPECTIVE STUDY OF THE NATURAL HISTORY OF DIABETES MELLITUS IN THE GILA RIVER INDIAN COMMUNITY

PREVIOUS SERIAL NUMBER: NIAMD-EFS-1(c), 2(c), & 5(c)

PRINCIPAL INVESTIGATOR: Dr. Peter H. Bennett

OTHER INVESTIGATORS:	Dr. Thomas A. Burch Dr. Leonard J. Comess Dr. Robert E. Henry Dr. R. Gordon Senter Dr. Max Miller Dr. Arthur Steinberg Dr. Norman Rushforth Dr. Elmer J. Ballantine Dr. Irving M. Liebow Dr. Fredrick A. Rose Dr. Henry W. Blackburn, Jr. Dr. Marion E. Black
COOPERATING UNITS:	Division of Indian Health, PHS Case Western Reserve University University of Minnesota

MAN YEARS:

Total	10
Professional:	3
Other	7

PROJECT DESCRIPTION

Objectives:

1. Determine the prevalence and incidence of chemical and clinical diabetes mellitus among those 5 years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from prediabetic state to chemical diabetes mellitus and to clinical diabetes.

3. Study the association between diabetes and the rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age, obesity, parity, and other

factors on changes in glucose tolerance and their relationship to diabetes mellitus.

Methods:

1. The following tests and examinations will be repeated on all Indians 5 years of age or more living on the Sacaton Service Unit portion of the Gila River Reservation:

a. Clinical interview
b. Physical examination
c. Modified glucose tolerance test
d. Fundus photograph
e. Electrocardiogram
f. Test for vibratory sensation threshold
g. Soft tissue radiographs for vascular calcification.

2. Some of the above tests and examinations will also be done on Indians of other tribes or living under different environment conditions for comparison.

Major Findings:

1. The prevalence of diabetes. The prevalence of diabetes in the Gila River Indians has been previously reported. The medical records of all those aged 15 years and over who had not received glucose tolerance tests were sought and the medical records of 89 percent of the nonresponders were identified and reviewed for the presence of a clinical diagnosis of diabetes. The prevalence of known diabetes in the responders and nonresponders was compared.

The results of this investigation indicate that the responders are closely representative of the total sample and that there is no reason to suppose that the failure to examine some of them has prejudiced the findings in any significant way.

PREVALENCE OF KNOWN DIABETES IN GILA RIVER INDIANS
(RATES/100 PERSONS)

	MALES		FEMALES	
Age Group	In Persons Examined	In Total Sample	In Persons Examined	In Total Sample
15-24	0.0	0.0	0.0	1.2
25-34	1.3	3.4	7.7	6.3
35-44	11.2	14.9	16.0	17.1
45-54	19.2	19.7	26.6	32.7
55-64	24.2	28.1	44.3	43.8
65+	20.0	20.6	22.2	20.7
TOTAL	11.1	10.5	14.9	14.3

2. Birth weights of Pima children. Birth weights of 835 of the 1252 children of Pima Indian women aged 25 to 44 when tested at the NIAMD Clinic at Sacaton have been analyzed as a function of the diabetic status of the mother.

Maternal Diabetic Status	No. Of Children	Birth Weight 10# and over No.	%	Mean Birth Weight ± S.E.M.
Nondiabetic (GTT < 140mg%)	571	7	1.2	7.47 ± .05
Prediabetic (Prior to diagnosis of diabetes)	229	11	4.8	7.72 ± .08*
Diabetic (After diagnosis of diabetes)	35	9	25.7	8.54 ± .29**

* Higher than nondiabetics ($p < .05$)
** Higher than prediabetics ($p < .05$)

3. Perinatal Mortality in Pima. Preliminary results of the record review of the 1252 children of Pima Indian women aged 25-44 when tested at the NIAMD clinic at Sacaton indicate, as expected, that the children of diabetic mothers have a much higher perinatal mortality rate than children of nondiabetics. Although diabetic pregnancies comprised less than 5% of all pregnancies, they resulted in 32% of all perinatal deaths.

Maternal Diabetic Status During Pregnancy	All Pregnancies	Stillbirths No.	%	Neonatal Deaths No.	%	Perinatal Mortality No.	%
Nondiabetic	813	5	0.6	5	0.6	10	1.2
Prediabetic	392	9	2.3	6	1.5	15	3.8
Diabetic	47	6	12.8	6	12.8	12*	25.5
Totals	1252	20	1.6	17	1.4	37	3.0

*$p = 0.001$

4. Congenital Anomalies. Children from "diabetic" pregnancies had more congenital anomalies than those from "nondiabetic" or "prediabetic" pregnancies. The low frequency of anomalies from pregnancies of prediabetic women and in children of diabetic and prediabetic men suggest that, rather than being genetically determined, the anomalies were produced by the effect of maternal diabetes on the intrauterine environment. Consistent with this was the higher frequency of anomalies from pregnancies during which insulin was needed to control maternal diabetes.

Maternal Diabetic Status	All Children	Children with Anomalies No.	%
Nondiabetic	785	31	4.0
Prediabetic	375	13	3.5
Diabetic	47	9*	19.1
Totals	1207	53	4.4

* $p = < 0.001$

Paternal Diabetic Status	Maternal "diabetic" Pregs. Excluded		
	All Children	Children with anomalies No.	%
Nondiabetic	395	13	3.3
Prediabetic	197	7	3.6
Diabetic	44	2	4.5
Total	636	22	3.5

5. *Prospective Study of Diabetes and Pregnancy*. A total of 526 Pima or Papago women have been delivered at Sacaton or Good Samaritan Hospital since the onset of the study in October 1965. Analysis indicated that

a. Toxemia of pregnancy was associated with increasing maternal age, *not* with increasing parity, and possibly with increasing postpartum plasma glucose.
b. Infant birth weight was associated with increasing maternal age and/or weight and was independently associated with increasing postpartum glucose.
c. Maternal weight varied directly with maternal age. It was *not* associated with postpartum maternal glucose.
d. Maternal postpartum glucose was not associated with either maternal age or maternal weight.

6. *Blood Pressure*. Preliminary analyses of blood pressure data indicated that the mean systolic blood pressures rose with increasing age in both sexes. The magnitude of the rise was similar in both males and females but in the males was much less marked than in samples representative of the United States. The characteristics of the mean diastolic blood pressure levels were similar.

When the findings among the diabetics were contrasted with those of the total population the diabetics tended to have levels of systolic and diastolic pressures which were only slightly, but consistently, higher than the age-sex specific population means. The possible reasons for these differences will be explored, and the temporal sequence of the relationship will be determined in the ongoing prospective study of these characteristics.

7. *Serum Cholesterol*. Serum cholesterol levels among the Gila River Indians were found to be lower than those in the general U. S. population. The levels in the males up to age 54 years were higher than in those aged 55 years and over. In contrast the mean levels in the females increased steadily up to age 60 years but were lower in those aged over 65 years.

The mean levels of the diabetics in all age-sex groups were not significantly different from the means of the total population of the same age and sex.

8. *Electrocardiographic Changes*. Standard twelve lead electrocardiograms were performed on approximately 1,000 Gila River Indians aged 15 years and over. The presence of coronary artery disease was evaluated by reading each record according to the Minnesota reading code without knowledge of the age, sex, or diabetes status of the subject.

In the Pima Indians the major electrocardiographic abnormalities were slightly more common in the females than in the males. This difference was most marked in those aged 55-64 where major abnormalities were twice as frequent as in the males.

AGE-SPECIFIC PREVALENCE OF MAJOR ELECTROCARDIOGRAPHIC ABNORMALITIES IN THE GILA RIVER INDIANS

	MALES			FEMALES		
	NUMBER	ABNORMAL		NUMBER	ABNORMAL	
AGE	EXAMINED	NO.	%	EXAMINED	NO.	%
15-24	112	7	6.3	126	11	8.7
25-34	50	2	4.0	81	2	2.5
35-44	77	4	5.2	128	9	7.0
45-54	62	6	9.7	78	8	10.3
55-64	63	6	9.5	74	15	20.3
65+	87	17	19.5	57	12	21.1
	451	42	9.3	544	57	10.5

In order to assess the relationship of electrocardiographic findings the plasma glucose levels of each age group were divided into three groups by quintiles, the upper two quintiles, the second two, and the lowest quintile.

EKG CHANGES AND RELATIVE PLASMA GLUCOSE LEVEL
(RATE/100 PERSONS)

EKG	PLASMA GLUCOSE							
CHANGES	MALES				FEMALES			
	LOW	MED	HIGH	P	LOW	MED	HIGH	P
ST	1.7	3.4	8.6	<0.05	0.0	3.5	3.5	NS
T	5.3	7.7	14.7	<0.05	8.3	16.2	9.1	NS
BBB	3.5	1.7	3.5	NS	0.0	2.8	5.6	<0.05
ANY CHD	8.8	12.1	21.6	<0.05	8.3	19.0	14.7	NS

9. Diabetic Neuropathy. Approximately 1000 Gila River Indians were tested for vibratory sensation threshold using a Bio-thesiometer in the period March 1965-March 1967. Evidence of severe impairment of vibratory sensation in the fingers and toes was found in 183 persons.

In 64 of those subjects there was evidence that this impairment was directly related to diabetes. Among the remainder, 32 were possibly associated with the presence of diabetes.

Thus, among the Gila River Indians, diabetes appears to be the most frequent cause of sensory impairment. Further analyses will be made to define the associated conditions and attempts will be made to identify specific etiologic factors in addition to related characteristics of the associated diabetes.

10. Bimodal Plasma Glucose Frequency Curves. Plotting the frequency distribution of the two-hour plasma glucose values using log of the values gave a bimodal curve for all age and sex groups tested except males aged 15-24. This is compatible with the hypothesis that diabetes or susceptibility to diabetes is carried by a single gene.

11. Diabetes Family Study. When the diabetes status of the father and mother was considered separately, there was definite familial aggregation

when the mother had diabetes but not when the father had diabetes. The frequency of diabetes in families with father only diabetic was the same as in families with neither parent diabetic.

DIABETIC STATUS* OF PARENTS		OFFSPRING		
Father	Mother	Examined	Diabetic	Rate of Diabetes Adjusted for Age and Sex
+	+	131	34	20.7
0	+	114	18	15.3
+	0	107	9	10.8
0	0	152	9	10.8

* Diabetes = known treated diabetics or two-hour post loading plasma glucose of ≥ 140mg%.

Significance of Research:

The remarkably high frequency of diabetes among the Gila River Indian Community has provided the opportunity to evaluate factors associated with the disease and its complications in a strictly controlled manner. Such observations enable a large variety of hypotheses concerning possible etiologic factors to be explored. The prospective study will enable the sequence of events which lead to the development of diabetes and subsequently to its complications to be determined. It is hoped that such information may lead to the development of measures which may delay or prevent the appearance of the complications or of the manifestations of the basic disease.

Proposed Course:

The prospective study of diabetes among the Pima Indians will continue. The frequency of complications will be determined and associated factors will be sought. Groups at high risk of development of diabetes will be identified and studied repeatedly to clarify the role of obesity and other factors in relation to diminished carbohydrate tolerance and insulin secretory function.

The significance of the glucose tolerance test and its variability will be investigated in relation to the development of major and microvascular complications.

HONORS AND AWARDS: None

PUBLICATIONS:

Genuth, S. M., Bennett, P. H., Miller, M., and Burch, T. A.: Hyperinsulinism in obese diabetic Pima Indians. Metabolism 16: 1010-1015, Nov. 1967.

Miller, M., and Bennett, P. H.: Epidemiology of Diabetes. In Hamwi, G. J., and Danowski, T. S. (Eds.): Diabetes Mellitus: Diagnosis and Treatment. New York, American Diabetes Association, 1967, Vol. 2, pp. 13-19.

Serial No. NIAMD-EFS-6(c)
1. Epidemiology & Field Studies Br.
2. Clinical Field Studies Unit
3. Phoenix, Arizona

PHS-NIH
INDIVIDUAL PROJECT REPORT
July 1, 1967 through June 30, 1968

PROJECT TITLE: DIET AND NUTRITION OF THE GILA RIVER INDIANS

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Dr. Thomas A. Burch

OTHER INVESTIGATORS:
Dr. Peter H. Bennett
Miss Jeanne M. Reid
Dr. G. Donald Whedon
Dr. Max Miller

COOPERATING UNITS:
Division of Indian Health (PHS)
Case Western Reserve University

MAN YEARS:
Total: 1 1/3
Professional: 2/3
Other: 2/3

PROJECT DESCRIPTION:

Objectives:

1. To ascertain whether there is an association of dietary characteristics with the presence of diabetes mellitus, choleliathiasis, or rheumatic conditions in the Gila River Indian Community.

2. To ascertain whether there is an association of dietary characteristics with the development of such conditions during the next ten years.

Methods:

1. Samples of the Gila River Indian Community being followed in the various CFSU programs on diabetes, choleliathisais and rheumatic diseases will be interviewed to ascertain their dietary characteristics.

2. The medical status of the participants will be determined at periodic intervals and possible association of dietary characteristics and development and/or progression of such conditions evaluated.

Major Findings:

The first phase of the field studies has been completed. This consisted of obtaining diet interviews on the Pima women age 25 through 44 living on the Sacaton Service Unit area of the Gila River Indian Reservation. Coding and tabulation of the data has not been completed and there are no findings to report at this time.

Significance of Research:

Diet plays an important part in both diabetes mellitus and gallbladder disease as demonstrated by control or alleviation of symptoms of these diseases in many patients by means of dietary regulation. This suggests that a study of the diet of affected and nonaffected individuals may contribute to an understanding of their etiology.

Proposed Course:

The data from the diet interviews as well as the medical and other pertinent data will be analyzed by means of computer programs to evaluate dietary differences and possible association with the individuals medical status.

HONORS AND AWARDS: None

PUBLICATIONS: None

METABOLIC DISEASES EPIDEMIOLOGY UNIT
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

SUMMARY

The long-term objectives of the program are: (a) to examine closely the principal environmental factors associated with prevalence of goiter and (b) to study the nature of the metabolic defect responsible for the disease.

Preliminary studies of the relationship of intestinal flora of man and rat were continued, particularly in an effort to determine whether or not colonization of the rat intestine could influence Thyroid function and, in man, whether or not the appearance of Clostridium perfringens in the intestinal tract was associated with hyperthyroidism. At this time the results are inconclusive but there is a suggestion that a semi-purified thyroid stimulating factor has some activity in the rat. Further studies of the relationship are planned.

Serial No. NIAMD-EFS-7(c)
1. Epidemiology & Field Studies Br.
2. Metabolic Diseases Epidemiology Unit
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title:	Field Studies of Iodine Metabolism
Previous Serial Number:	NIAMD-EFS-6(C)
Principal Investigators:	Robert L. Vought, M.D., M.P.H.
Other Investigators:	Christian Beckers, M.D. Freddie A. Brown, B.S.
Cooperating Units:	Metabolic Diseases Branch; Clinical Endocrinology Branch; Laboratory of Nutrition and Endocrinology, NIAMD; Radiation Safety Office, NIH; Instrument Fabrication Section, NIH; Theoretical Statistics and Mathematics Unit, Biometry Branch, NIMH; Richmond County Virginia Health Department; Bionetics Research Laboratory (research contract).

Man Years

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives: The long-term objectives of the program are: (a) to examine closely the principal environmental factors associated with prevalence of goiter and (b) to study the nature of the metabolic defect responsible for the disease.

Methods Employed: Accomplishment of the objectives has involved bench laboratory research, clinical research and collaborative field studies.

In the laboratory techniques are being developed for the quantification of bacteria from the human and rat intestinal tracts, and for the quantification and identification of *Clostridium perfringens* and their exotoxins. The use of immunodiffusion and immunoelectrophoresis techniques for the recognition of the antigens and antibodies to *Clostridium perfringens* are being developed, with the help of the Bionetics Research Laboratories contract. In cooperation with the Laboratory of Nutrition and Endocrinology, NIAMD, animal studies have been carried out attempting to relate changes in thyroid function to changes in the intestinal flora of the rat. Considerable effort has been expended in studying the toxicology of the Pastan semi-purified thyroid stimulating factor (TSF) in the rat. In cooperation with the Clinical Endocrinology Branch, iodine balance studies in hyperthyroid girls, fecal iodine output in hyperthyroidism and a comparison of intestinal flora in hyperthyroid patients *vis-as-vis* normal controls have been carried out. Preliminary field studies have been made of anaerobic bacterial flora of shallow wells in Richmond County, Virginia. Through cooperation of the Theoretical Statistics and Mathematics Unit, NIMH, iodine balance data from Bethesda and Louvain, Belgium, are being used to develop a computerized iodine balance study program.

Major Findings: Studies have shown that in the rat inter-peritoneal injection of semi-purified TSF stimulates release of iodine from the thyroid and that this effect is blocked by *Clostridium perfringens* Type A antitoxin. Preliminary studies of the effect on the thyroid of oral feeding of *Clostridium perfringens* cultures to germ-free rats were negative on an acute basis (4 days or less). Studies of chronic feeding over a longer period of time are being done in conventional rats.

Four iodine balance studies in two hyperthyroid girls yielded convincing evidence that they were in a strongly positive iodine balance. During these studies it was noted that the output of fecal iodine could be extremely high on an episodic and very short term basis (one or two days).

Earlier work at NIH had indicated that negative iodine balance was the usual finding in euthyroid subjects on iodine intakes of 50-600 mg per day. Work in Louvain, Belgium, has yielded similar results in euthyroid subjects on intakes of 20-40 mg per day. Data from the two laboratories are being used to describe and investigate a computerized model of iodine intake, excretion and thyroidal accumulation.

Informal reports from Greece indicate that our finding of an association between goiter prevalence and polluted water is to be confirmed there, and one published report to that effect has appeared in the German medical literature.

This year studies have adduced additional evidence that there is some relationship between the intestinal flora and thyroid function. Further studies are needed to confirm these preliminary results.

Proposed Course of Project: The studies outlined above are to be continued next year with the objective of applying the newly developed techniques to clinical and field research.

Publications:

Malamos, B., Koutras, D.A., Marketos, S.G., Rigopoulos, G.A., Yataganas, X.A., Binopoulos, D., Sfontouris, J., Pharmakiotis, A.D., Vought, R.L., and London, W.T.: Endemic goiter in Greece: an iodine balance study in the field. *J. Clinc. Endocr.* 27:1372-1380, Oct. 1967.

Vought, R.L., London, W.T., and Stebbing, G.E.T.: Endemic goiter in Northern Virginia. *J. Clin. Endocr.* 27:1381-1389, Oct. 1967.

Vought, R.L.: Nontoxic goiter. In Wagner, Jr., H.W. (Ed.): Principles of Nuclear Medicine. Philadelphia, W.B. Saunders Co., 1968, pp. 330-332.

INFORMATION OFFICE ACTIVITIES

Summary

Fiscal Year 1968

The Information Office conducts the Institute's public information and educational programs, and prepares the bulk of the regular and special program reports required of the Institute by the Congress, and by NIH and DHEW.

Both the scope and depth of the Institute's categorical research programs have increased rapidly in recent years, and reporting on all of these varied and complex activities has made heavy demands on the information staff. Accompanying this growth has been an increase in the number of internal reports required of this Institute by the Congress and by the Department.

Press Releases and Articles:

Fiscal 1968 saw a marked increase in the number of press releases as compared to the previous fiscal year. Noteworthy releases included the awarding of a new contract to the National Pituitary Agency, which afforded the press an opportunity to review progress in dwarfism and human growth hormone research, and the revelation that gallbladder disease is six times more common among the Pima Indians than it is among Caucasians.

Two other press releases also deserve special recognition. These concerned the publication of two new informational pamphlets on psoriasis and systemic lupus erythematosus. The resulting avalanche of requests from the public for individual copies of these publications led to the employment of one additional staff clerical member and to an approximate 50 percent increase over FY 1967 in the number of informational publications distributed by the Information Office.

Also of significance was the preparation of four different articles on artificial kidney, diabetes, arthritis, and osteoporosis research for Today's Health, a lay publication with a circulation of more than one million. These articles, signed respectively by Drs. Benjamin T. Burton, R.W. Lamont-Havers, John Decker, and G. Donald Whedon, were later issued as reprints for the Information Office to distribute in response to public inquiries.

NIAMD Research for Health columns, written specifically for a large number of small newspapers throughout the country, concerned the latest research findings in such fields as artificial kidneys, metabolic diseases, gastroenterology, human growth hormone, and diabetes. The impact of these articles is evidenced not only by NIH access to several newsclipping services, but also by an increasing number of queries directed to the Information Office concerning specific articles.

Radio and Television:

Inroads into the media of television continue to be made. In Fiscal 1968 a

CBS news team filmed ongoing Institute diabetes research among the Pima Indians for use on Walter Cronkite's 21st Century television program. The Information Office coordinated this operation and provided background material on diabetes research. As of this writing, this program has not yet been televised.

Assistance also was provided to an NBC television film crew in filming NIAMD research advances in genetics and metabolic diseases studies on the NIH campus for an upcoming edition of the Tomorrow's World television program. In addition, information was provided on NIAMD nutrition studies in this country and abroad for an NBC television "white paper" on nutrition, food, and starvation.

In radio, NIAMD's Dr. Paul di Sant'Agnese discussed the latest research findings in cystic fibrosis on the Metromedia network, an event which was coordinated by Information Office and Metromedia personnel. One Information staff member addressed a local California radio audience on the Institute's support of the National Pituitary Agency and human growth hormone research.

Press Briefings:

Two highly successful press briefings, on diabetes and on kidney disease, were conducted during the past fiscal year. The former featured NIAMD's Dr. Jesse Roth, who expanded on the theory that diabetes may be primarily a disease of the vascular system, rather than an abnormality of carbohydrate metabolism. Dr. Benjamin T. Burton briefed the press on the latest developments in the fields of artificial kidney research and development and kidney disease.

These press briefings involved many man-hours of work by the Information Office staff devoted to the preparation of press kits to include photographs of the speakers, biographies, highlights of each presentation, background material, and several press releases.

Voluntary Foundations and Other Private Groups:

The maintenance of good public relations with a number of voluntary organizations is a continuing function of the Information Office. During FY 1968, for example, Information Office personnel helped staff the press room during the annual meeting of the American Rheumatism Association. A press release was issued jointly by the Information Office and the National Cystic Fibrosis Research Foundation (NCFRF) announcing the completion of a new medical teaching film, "Diagnosis and Management of Cystic Fibrosis," which was produced under NIAMD-NCFRF auspices. At the request of the Federation of American Societies for Experimental Biology (FASEB), non-technical abstracts of papers to be read by NIAMD scientists at the Federation's 52nd annual meeting were prepared for distribution to news media representatives.

Publications and Reports:

The constant increase in NIAMD research programs has also been felt in the publications and reports section of the Information Office, which produced more new or revised publications during FY 1968 than during any previous comparable period. Two new publications in particular, "Psoriasis" and

"Systemic Lupus Erythematosus", have proved extremely popular, and Information staffers have been hard-pressed meeting the demands for same. Another popular new publication is a fact sheet about "Hypoglycemia". Publications which were revised and updated included "Facts About Nutrition" and "Facts About Cystic Fibrosis."

Three new publications presently are being prepared and these should be available within the next fiscal year. They are tentatively labeled "Gout", "Kidney Disease and The Artificial Kidney", and "Research in Arthritis."

The Weekly Report to the NIH Director is designed to inform the Director of current NIAMD scientific achievements, both intramural and extramural, and to inform other DHEW professional personnel of noteworthy developments outside of their own specific areas of interest. Characteristic Weekly Reports during the past fiscal year concerned the successful transplantation of livers in four young girls; development of a new technique for storing and re-using conventional artificial kidney equipment; identification of the specific biochemical defect in four comparatively rare metabolic diseases, and identification of a similar defect in some patients with gout.

In addition, as developments warranted, similar reports on current or planned program developments were prepared for the NIH Director.

Congressional Testimony:

A major effort of the Information Office each year is the preparation of the series of special reports and statements involved in the annual NIH budget presentation. This collection of materials consisted in FY 1968 of the following:

1. Director's Opening Statement
2. Highlights of Progress in Research on Arthritis
3. Special Report on Diabetes
4. Special Report on Arthritis
5. Special Report on Gastroenterology
6. Special Report on Cystic Fibrosis
7. Special Report on Kidney Disease
8. Budget Narrative

It is anticipated that several other special reports will be required by the Congress during the current session, inasmuch as such a pattern has been followed in previous years. Such reports invariably have tight deadlines and call for a concerted effort by Information Office staff members.

Public Inquiries:

Inquiries from the general and scientific publics take many forms. Each, however, receives a prompt individualized reply, which usually is supplemented by written informational material. If, as often happens, a referral to another agency for information is indicated, this is done swiftly and courteously.

During fiscal year 1968 Information Office personnel responded to 1,925 public inquiries (telephone and letter), 99 congressional inquiries (telephone and letter), and distributed 22,744 publications of an informational nature. In addition, 188 inquiries from various news media were handled with dispatch. All of these figures are up markedly from FY 1967.

Exhibits:

The Information Office's major effort in the field of professional education is the scientific exhibits program. This past year, the Information Office completed yet another strong and varied exhibit schedule. NIAMD showed seven different scientific exhibits at 14 medical and scientific meetings throughout the country which were viewed by about 115,000 physicians, scientists and paramedical personnel. Institute exhibits on human growth hormone, gout, arthritis, artificial kidney, and current awareness publications constituted the bulk of items making the viewing circuit. NIAMD exhibits have won many awards and elicited much newspaper and radio publicity and complimentary comment by physicians and others who have viewed them.

Some of the medical meetings where exhibits were shown include the New York and Massachusetts Medical Societies, the annual and clinical sessions of the American Medical Association, the American Public Health Association, the American Academy of General Practice, The American Society for Artificial Internal Organs, the American Urological Association, and others.

A new NIAMD exhibit on intramural studies of Lesch-Nyhan disease was completed during this past year. Developed by the Information Office, in cooperation with Dr. J. E. Seegmiller and the NIH Medical Arts Section, the exhibit will make its debut at the June, 1968 meeting of the American Medical Association. Three other exhibits were revised along with the accompanying literature.

NIAMD is one of the most active Institutes in the exhibit area, having shown more than 20 percent of all NIH exhibits presented in 1967.

OFFICE OF THE DIRECTOR

Summary

Fiscal Year 1968

Dr. G. Donald Whedon, Director
Mr. W. G. Baylis, Executive Officer

The Office of the Director has overall responsibility for directing and coordinating basic laboratory research, clinical investigations, extramural activities such as research grants, research fellowships, and training grants, collaborative contract programs and clinical field studies. In addition, this Office cooperates with other scientific organizations in coordinating medical research in the Institute's fields of interest; cooperates and maintains liaison with other Institutes and constituents of other Federal agencies; and participates in determining policy governing the National Institutes of Health. It is also responsible for the collection and dissemination of informational material to interested professional and lay individuals and groups.

Several changes have been made in the staffing of some of the key positions within the Institute during this fiscal year. Dr. G. Gilbert Ashwell has been appointed as Chief of the Laboratory of Biochemistry and Metabolism, Dr. Robert G. Martin has been appointed as Chief of the Section on Microbial Genetics, Laboratory of Molecular Biology, Dr. Ned Feder as Chief of the Section on Biophysical Histology, Laboratory of Experimental Pathology, Dr. William B. Jakoby as Chief of the Section on Enzymes and Cellular Biochemistry, Laboratory of Biochemistry and Metabolism.

The activities of the Institute's Information Office are included in a separate report which precedes this summary. It is noteworthy that this year the Information Office has increased efforts aimed at informing the general lay public of the Institute's mission and activities. The distribution of over 15,000 information brochures requested by the public and a series of disease-related articles in "Today's Health" were a part of these efforts.

Scientific and administrative overview of the Clinical Field Studies Unit in Phoenix, Arizona is carried out by the Director and Executive Officer. This unit is conducting studies on Indian populations - predominantly the Pima tribe on the Gila River Indian Reservation - on arthritis, diabetes, nutritional abnormalities and gallbladder disease. These 10-15 year studies will include hereditary, environmental, dietary, and anthropological aspects, that may have a bearing on the cause of these diseases. The activities of the Clinical Field Studies Unit are included in a separate report which precedes this summary.

The Program Contract Office entered FY 1968 with 73 ongoing contracts and PL-480 agreements. These include contracts for intramural research requirements, scientific communications, such as journals and abstracts, artificial kidney research and development, and hormone distribution programs.

During this fiscal year 67 contract proposals with a face value of over $5 million were received and evaluated. Approximately thirty new contracts were awarded during the year.

Management emphasis continues to be placed on enhancing the short-term and long-term training opportunities offered both in-service and through nongovernmental facilities. During the year specialized short-term training opportunities were arranged for 44 secretarial-clerical employees, 18 supervisory managerial personnel, and 97 members of the professional staff. In addition, this year, one of our employees was selected for long-term training in a non-Government training facility under the National Institute of Public Affairs for the Mid-Career Education Program in systematic analysis.

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